PROLIFERATIVE AND CYTOTOXIC CELLULAR IMMUNE RESPONSES IN HUMAN TUBERCULOSIS

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FOR MY FAMILY

and for all oppressed people in South Africa

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ABBREVIATIONS USED IN THIS THESIS

AB serum,	pooled allogenic AB serum
Ag,	antigen
AIDS,	acquired immune deficiency syndrome
APC,	antigen presenting cell
BAL,	broncho-alveolar lavage
BCG,	bacille calmette geurin
CFA	Complete Freund's adjuvant
CI,	confidence interval
cpm,	counts per minute
CTL,	cytolytic/cytotoxic T lymphocyte
df,	degrees of freedom
E/T,	effector to target ratio
EDTA,	ethylene diamine tetra-acid disodium
FCS,	foetal calf serum
Fig,	figure
FITC,	fluorescein iso-thiocyanate conjugate
HIV,	Human Immunodeficiency Virus
HS,	human serum
HSP,	heat shock protein
IFN-δ,	interferon-gamma
IL-2,	interleukin-2
LAK,	lymphokine activated killer
LAM,	Lipoarabinomannan
LPS,	lipopolysaccharide
M. bovis,	*Mycobacterium bovis*
M. tuberculosis,	*Mycobacterium tuberculosis*
mg,	milligram
MHC,	major histocompatibility complex
ml,	milliliter
NAC,	non-adherent cell
ND,	not done
NK,	natural killer
PAT,	patient
PBL,	peripheral blood lymphocyte

PBMC,	peripheral blood mononuclear cell
PBS,	phosphate buffered saline
PEL,	pleural effusion lymphocyte
PHA,	phytohaemagglutinin
PPD,	purified protein derivative of *Mycobacterium tuberculosis*
SD,	standard deviation
SDS-PAGE,	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF,	synovial fluid
SK-SD,	streptokinase-streptodornase
SRBC,	sheep red blood cell
SUP,	supernatant
TB,	tuberculosis
TNF,	tumor necrosis factor
µci,	microcurie
µg,	microgram
µl,	microliter

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SYNOPSIS

Several aspects of the cellular immune response in patients with tuberculosis were examined.

A subset of patients were noted to have persistent spontaneous peripheral blood lymphocyte proliferation as assessed by *in-vitro* ^{3}[H]thymidine incorporation. Receptor studies (i.e. identification of the interleukin-2 receptor by immunostaining and dose-dependent blocking with anti-interleukin-2-receptor antibody) confirmed that the proliferation was interleukin-2 dependent. The kinetics of the proliferation, the strict requirement for accessory cells (as determined in cell separation experiments) and the fact that adherent cell supernatants obtained from these patients significantly stimulated cells from control PPD responders suggested that persisting mycobacterial antigen was responsible for the phenomenon.

Since cytotoxic T cells have been invoked as an important protective mechanism against Mycobacteria, evidence for their induction was sought in patients with tuberculous pleuritis. PPD-specific cytotoxicity in tuberculous pleural effusion lymphocytes was found to be augmented and accelerated when compared to autologous peripheral blood, control peripheral blood or non-tuberculous pleural effusions. The cytotoxicity could only be demonstrated in 15-hour chromium release assays. Cell subset enrichment by panning indicated that the cytotoxicity was mediated by $CD4^{+}$ cells. The cytotoxicity was MHC class II restricted. Antigen non-specific cytotoxicity was also noted and found to be due to NK cells. All attempts to demonstrate PPD-specific cytotoxicity in conventional 4-hour chromium release assays failed. This included experiments utilizing cytoplasmic loading by osmotic lysis of pinosomes in order to introduce

soluble PPD or BCG into the class I pathway of antigen processing.

The proliferative response of pleural effusion lymphocytes to PPD was also found to be accelerated. The diagnostic value of this finding was evaluated. 65% of 31 patients with tuberculous pleuritis had an accelerated proliferative response. The phenomenon was not seen in non-tuberculous effusions.

Proliferative and cytotoxic responses of pleural effusion lymphocytes to the recombinant 65 kDa heat shock protein of Mycobacterium Bovis BCG were determined. A marked compartmentalization of the proliferative response was observed with a much higher response by pleural effusion lymphocytes as compared to autologous peripheral blood. Non-tuberculous effusions were not reactive. Pleural effusion lymphocytes stimulated with the 65 kDa antigen also showed antigen-specific lysis of autologous adherent target cells treated with the same antigen.

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

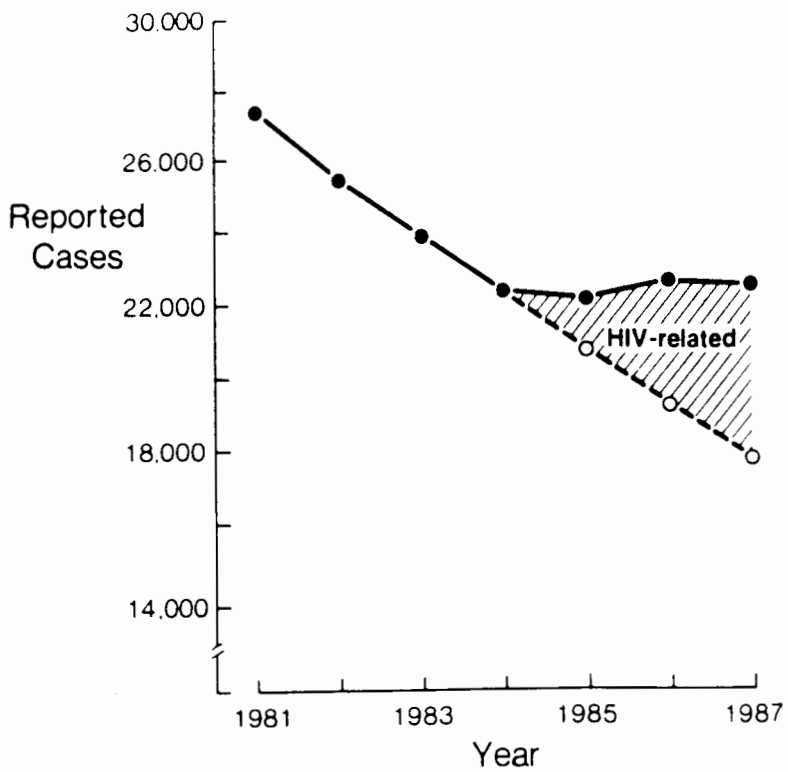
Tuberculosis is probably the oldest infection known to man (1). Reports of its existence date back to antiquity (1,2). Although much progress has been made, the disease continues to be a major problem worldwide (2,3). Developing countries carry the brunt of the burden, with Africa having the highest incidence (4). Approximately 10 million individuals are thought to be infected with the Tuberculosis bacterium in South Africa and the annual notification rate from 1981-1985 in the city of Cape Town (the region of highest incidence in South Africa) was 312-370 per 100 000 population (5,6). Notification rates in the developed countries are also on the increase as a direct result of the AIDS pandemic (Fig.1.1). This has resulted in a renewed demand for basic and clinical research in tuberculosis (2,3). Additional interest in the field of tuberculosis research has recently been generated by intriguing observations linking mycobacteria and autoimmunity (7).

1.2. THE IMMUNE RESPONSE TO INFECTION WITH MYCOBACTERIA

1.2.1. THE PATHOGENESIS OF TUBERCULOSIS

The hallmark of Tuberculosis is the formation of caseating granulomata. Lurie's fundamental studies with resistant and susceptible inbred rabbits have provided major insights into events leading up to granuloma formation (8). More recent advances have allowed clinical, histological, immunological and molecular correlates to be established (9). In Lurie's studies, the number of human-type bacilli was quantitated in the lungs of naturally resistant and naturally susceptible

Fig. 1.1. Graph showing the number of reported cases of tuberculosis in the United States from 1981 to 1987. Those that are HIV-related are shown by the hatched area. (Reproduced from reference 2)



rabbits after infection by quantitative airborne inhalation. On the basis of observations made during these studies, the pathogenesis of tuberculosis can be divided into four stages.

1.2.1.1. The First Stage

The first stage begins after the inhalation of the tubercle bacillus. An alveolar macrophage ingests the bacillus and often destroys it. This destruction depends on the inherent microbicidal power of the macrophage and the genetic and phenotypic virulence of the ingested bacillus. The microbicidal power of the alveolar macrophage is under genetic control and is also determined by its state of activation (10). Alveolar macrophages can be non-specifically activated by a variety of inhaled particles and their microbicidal power therefore exists before the inhalation of Tubercle bacilli. Specific T cell mediated immunity is therefore not required at this level of resistance.

1.2.1.2. The Second Stage

If the original alveolar macrophage fails to destroy or inhibit the inhaled bacteria, a symbiotic stage ensues. This stage is characterized by the logarithmic growth of bacilli and begins between 7 and 21 days after infection. Many blood borne macrophages enter the lesion. Histologically, macrophages of susceptible hosts contain more bacilli and are located in the alveoli. Macrophages in the lesions of the resistant host contain fewer bacilli and are usually located interstitially. Interestingly, these histological differences have no apparent effect on bacillary growth as the *rate* of bacterial multiplication is the same in resistant or susceptible rabbits. Macrophages in both types of host do not appear to be significantly microbicidal at

this stage and are not activated as they are usually B-galactosidase (a histo-chemical marker of macrophage activation) negative. It is evident therefore that the cytoplasm of blood-borne macrophages from both resistant and susceptible rabbits support bacillary growth equally well. This can be explained by the observation that genetic resistance to the tubercle bacillus is expressed only in activated macrophages (10).

1.2.1.3. The Third Stage

The third stage of the disease begins when the logarithmic bacillary growth stops abruptly. At this stage, approximately 2 to 3 weeks after inhalation of the bacilli, the host possesses cell mediated immunity. When the second stage ends, the lungs of susceptible rabbits contain 20-30 times more bacilli than do the lungs of resistant animals. In spite of the differences in bacterial load and inherent microbicidal power of the macrophages, the susceptible hosts inhibit bacillary growth as efficiently as the resistant hosts (8). Clearly, therefore, another mechanism must exist. This is evident from tissue sections of the lesions. The logarithmic growth stops as soon as caseous necrosis develops. A tubercle forms with a solid caseous center within which the now extracellular bacilli cannot multiply. Around this center, immature macrophages permit intracellular bacillary multiplication, but mature activated macrophages (produced by T lymphocyte and macrophage interaction) inhibit such multiplication. The tubercle bacillus can survive for years in this solid caseous material, but it cannot multiply due to the anoxic conditions, reduced pH and the presence of inhibitory fatty acids (11). Caseous necrosis appears to be mediated chiefly by cytotoxic T cells (12). Other factors including cytokines [particularly tumor necrosis factor (TNF)], reactive oxygen and nitrogen intermediates also seem to be involved (13,14).

The stationary phase of bacillary growth lasts at least 8 weeks. The difference in macrophage activation determines the subsequent course of the disease. In the resistant rabbits, the lesion becomes surrounded by numerous microbicidal macrophages. The bacilli that escape the caseous center are therefore ingested and destroyed. The tubercle becomes walled off, the caseous center inspissates and the disease is arrested, frequently for a lifetime. In the susceptible rabbits, the lesion is surrounded by many poorly activated macrophages, which still permit the bacillus to grow intracellularly (8,15). These macrophages die and the caseous center enlarges. With virulent bovine-type bacilli in susceptible rabbits, bacilli lodging in the draining tracheobronchial lymph nodes and elsewhere in the body are not destroyed. Multiple uncontrolled tubercles with extensive caseation form throughout the body, especially in the lungs, and the host may eventually die of the disease. With human-type bacilli, the disease in both strains of rabbits healed after many months. However, before such healing could take place, the susceptibles often developed metastatic haematogenous lesions while the resistants often developed cavities.

1.2.1.4. The Fourth Stage

Unfortunately, disease progression may occur even in resistant hosts. Such progression is caused by liquefaction and cavity formation (the fourth stage) (8,11,15-17). The factors that cause liquefaction are largely unknown (9). Hydrolytic enzymes and TNF may be involved (9,13). Characteristically, liquefied caseous foci and cavities do not occur in susceptible animals and are not common in infants and immunocompromised individuals (8,15). It thus appears that an intact cell mediated immune response is a pre-requisite for this stage. The liquefied material is an excellent growth medium for the bacilli. For the first time

they multiply extracellularly, often reaching tremendous numbers (9). A large antigenic load is produced that is rich in tuberculin-like bacillary products (9). Since these products are toxic to the tissues of the tuberculin-positive host, the walls of nearby bronchi often become necrotic and rupture, forming the cavity. The bacilli are then discharged into the airways, thereby reaching other parts of the lung, as well as the outside environment. Liquefaction therefore perpetuates the disease. Another consequence of the large numbers of bacilli is that antibiotic-resistant mutants may arise (9).

The progress of the disease in humans closely resembles that in rabbits (8,11,15-17). Significant differences do however exist, particularly with regard to the microbicidal power of macrophages, and aspects of these are detailed below.

1.2.2. T-MONOCYTE INTERACTION AND MACROPHAGE MEDIATED PROTECTION

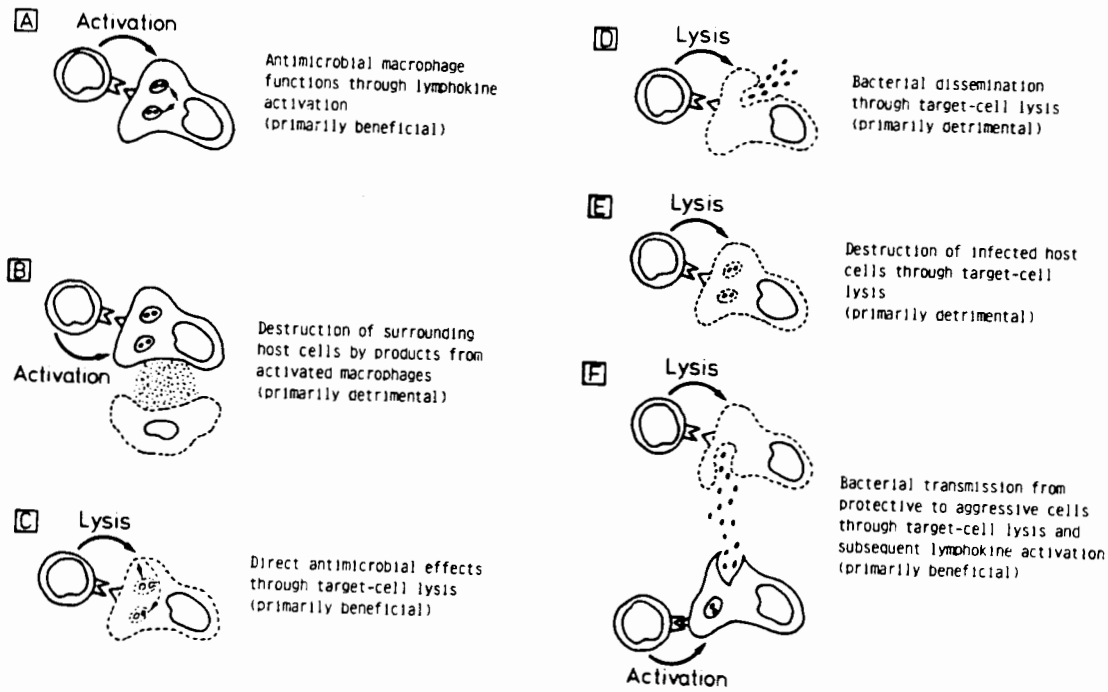
Intracellular bacteria including the etiologic agents of tuberculosis, *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Mycobacterium bovis* (*M. bovis*), are capable of surviving inside professional phagocytes (18). In this way they are well shielded from humoral defence mechanisms. It is thought that during their intracellular lives, mycobacteria produce molecules which are processed and presented in the context of major histocompatibility complex (MHC) products on the surface of infected macrophages (18). In this way host cells infected with mycobacteria are recognized by T lymphocytes. The importance of T lymphocytes in host defence has been established beyond doubt in both *in-vivo* and *in-vitro* studies (19-25). T lymphocytes interacting with infected host cells become activated and, in turn, produce cytokines which function to activate both T lymphocytes and macrophages (18). Interferon-gamma (IFN-ϑ) is thought to be the most important macrophage activating factor (26-28). In

man an additional pathway involving vitamin D3 further amplifies macrophage activation (29). Thus when human (but not murine) macrophages are exposed to IFN-ϑ they develop a 1-hydroxylase which converts the circulating inactive form of vitamin D3 (25-OH cholecalciferol) into the active 1-hydroxylated derivative [1,25 $(OH)_2$ D_3 or calcitriol]. Numerous animal studies have shown that macrophages activated with IFN-ϑ are tuberculostatic (28,30,31). The situation with human monocytes and alveolar macrophages is much less convincing (30-34). Human macrophages stimulated with IFN-ϑ and calcitriol show increased resistance to *M. tuberculosis* (35,36). *M. tuberculosis* and *M. avium-intracellulare* have however been shown to exhibit *in-vitro* resistance to the antimicrobial effects of IFN-ϑ treated human macrophages (31,33,34). In addition, studies have shown that IFN-ϑ may *accelerate* the growth of mycobacteria in human macrophages (30,37). In view of these equivocal observations, alternative mechanisms of protection are being investigated. Cytolysis of parasitized macrophages by specific T cells is an alternative mechanism, increasingly being considered, responsible for protection against mycobacterial infection (12).

1.2.3. CYTOLYTIC T CELLS IN MYCOBACTERIAL INFECTION

The hypothesis that cytolytic T cells (CTL) are an important effector mechanism is an attractive one. Lysis of parasitized host cells could have a number of either advantages or destructive consequences (Fig.1.2). Mere destruction of the cellular habitat may already be harmful to the microbe by exposing them to humoral effector mechanisms. Existence outside the cell may also be more difficult due to the possibility of dissolution by lysosomal enzymes (12). Also, lysis of infected host cells with low antimicrobial potential allows microbial transmission from a

Fig. 1.2. Possible consequences of T cell-macrophage interactions in intracellular bacterial infections. (Reproduced from reference 74)



protective niche to a more aggressive cell. Natural killer cells and lymphokine activated killer cells may also be able to kill the microbes directly (38-40). Destruction of parasitized macrophages could also minimize suppression since monocytes have been strongly implicated in this regard (1,41,42). It is also possible that destruction of infected host cells could lead to downregulation of the immune response by reducing the potential for antigen presentation and T-monocyte interaction.

Unfortunately, tissue destruction is an inevitable consequence of lysis. The stronger the immune response, and the more important and essential the target cell, the worse the consequences for the host (12). Another potentially destructive consequence of lysis is the threat of dissemination (12). Thus, secondary sites of the body may be colonized.

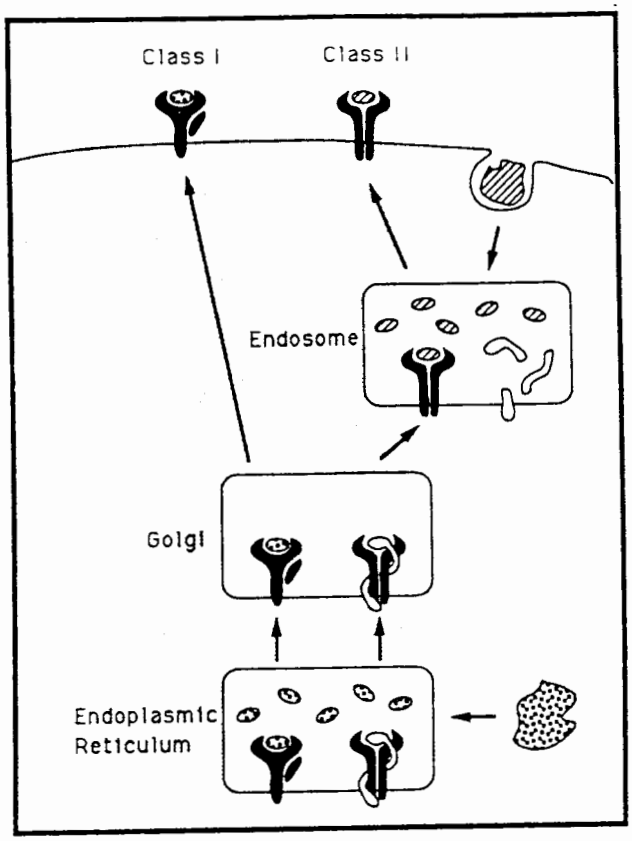
Experimental evidence supporting a role for CTL in protection against *M. tuberculosis* has recently become available (43-48). Specific CTL activity against mycobacterial infection has not been easy to demonstrate experimentally in humans. Only one study has demonstrated the presence of Purified Protein Derivative (PPD)-specific CTL using the conventional 4-hour chromium release assay (43). PPD-stimulated peripheral blood mononuclear cells (PBMC) were employed as effectors and targets were autologous PBMC labelled with PPD (43). Another study could only demonstrate significant lysis, in a 16-hour chromium release assay, when PPD was coupled to targets with the plant lectin Concanavalin A (44,45). No significant lysis was observed against targets labelled with PPD alone (44). Autologous Ebstein Barr virus transformed lines were used as targets. The low levels of kill observed in that study may have reflected an intrinsic property of the specific helper T cell clones employed as effectors (44). More recent

studies have been able to demonstrate lysis more readily by employing autologous adherent cell targets in 15-hour chromium release assays (46-48). All of these studies have demonstrated CD4 mediated MHC class II restricted lysis (46-48). Ultrastructural analysis of lesions in patients with leprosy has provided *in-vivo* evidence of lysis occurring at the site of the pathology (46). Similar evidence in tuberculosis has not been described to date.

It is now generally accepted that there are two distinct pathways of antigen processing and presentation (49-52). Exogenously introduced soluble proteins are presented by MHC class II molecules and endogenously synthesized antigens are presented by MHC class I molecules (49,51, Fig.1.3). The main functional distinction between the two MHC classes is that class I molecules interact with $CD8^+$ cytolytic cells and class II molecules interact with $CD4^+$ T cells with either helper or cytolytic function. Expression of MHC class II molecules, however, is restricted to certain cells with specialized antigen presenting functions, whereas virtually all other host cells express MHC class I molecules (52). It follows, therefore, that $CD8^+$ cytolytic T cells are likely to be required for lysis of most infected parenchymal host cells. Also, adoptive transfer experiments in mice have provided evidence implicating Lyt 2^+, class I restricted cells in protective immunity (24). Despite these theoretical arguments implicating a role for $CD8^+$ cytolytic T cells, supporting experimental evidence has not been produced in humans (12,53). Such evidence is only available in *in-vitro* murine studies utilizing Lyt-2^+ clones as effectors and bone marrow macrophages as targets (54).

The failure to demonstrate cytolytic activity mediated by $CD8^+$ cells in humans may be due to the inability to generate antigenic peptides able to enter the class I pathway of antigen processing. Exogenously added soluble protein

Fig. 1.3. Intracellular traffic and antigen processing. Different shadings have been used to represent MHC class I and class II molecules (black), invariant chain (white), exogenous antigen and fragments thereof (hatched), and endogenous antigen and fragments thereof (dotted). All elements are drawn schematically without any implication on their exact molecular structure.
(Reproduced from reference 52)



usually only enters the class II pathway of antigen presentation (52). The activation of $CD8^+$ CTL, however, requires antigen to be presented in the context of MHC class I (55). Recently it has been shown that soluble protein can be made to enter into the class I pathway of antigen presentation by the introduction of antigen directly into the cytoplasm of antigen presenting cells (55). EL4, a class II negative tumor target, was efficiently lysed by class I-restricted ovalbumin-specific CTL only after the introduction of antigen directly into the cytoplasm by osmotic lysis of pinocytic vesicles containing native ovalbumin (55). Similar results were obtained with CNBr digests of native ovalbumin (55). No lysis was observed when EL4 was labelled with either native or tryptic digests of ovalbumin (55). These data suggest that the form of the endocytosed antigen and its subcellular localization determine MHC class I versus class II presentation (55).

1.2.4. CYTOKINES AND TISSUE DAMAGE

The role of cytokines (particularly TNF) in tissue damage has been well documented in the necrotic response to intradermal challenge with antigens of *M. Tuberculosis* (13). Macrophages activated with IFN-ϑ and calcitriol show a high degree of priming for TNF release (29). In fact, macrophages obtained from alveolar lavage samples of tuberculosis patients spontaneously release TNF (56). *M. tuberculosis* is itself a potent trigger of cytokine release from primed macrophages (29). The major triggering molecule in *M. tuberculosis*, comparable in efficacy to LPS, is a phosphatidyl inositol mannoside known as Lipoarabinomannan (LAM) (57). *M. tuberculosis* not only triggers TNF release, but also renders its host's cells more sensitive to the toxicity of this cytokine, so that normally protective levels become detrimental to the host (13). The exact relationship of these observations which are characteristic

of the necrotizing skin response to the more slowly evolving caseous necrosis is not known (13). It is probable however that TNF plays a role in causing tissue damage in granulomatous lesions, particularly in Lurie's fourth stage where high bacterial loads are characteristic.

1.2.5. IMMUNE SUPPRESSION

Suppression of the cell mediated immune response is thought to play a role in the pathogenesis of tuberculosis (1,41,42). Monocytes, T lymphocytes, Natural killer cells and serum have all been shown to exert suppressor influences on the immune response to tuberculosis (1,41,42,58-61). However, all of the studies demonstrating suppression utilized cells obtained from the peripheral blood (1,41,42,58-61).

Age and poor nutritional status are also widely believed to negatively impact on the immune response to tuberculous infection (62-64). A potential mechanism for the decreased resistance observed in the elderly has been provided by experimental evidence showing a loss of protective T cell function in aged mice infected with *M. tuberculosis* (62).

1.2.6. PURIFIED ANTIGENS

Characterization of the antigenic components of mycobacteria is essential for the understanding of the immune response in patients, and could provide the basis of both serodiagnostic and skin tests. The most useful antigens for such tests would be recognized by many patients, would not be present in other mycobacteria and would be preferably immunodominant antigens. Progress in this field has been hampered by the scarcity of purified antigens. At least for protein antigens this problem has been solved because recently recombinant

DNA clones have been used to produce large quantities of pure mycobacterial protein antigens (65,66). Other techniques have also been employed to purify protein antigens. These include affinity chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (66).

1.2.6.1. The Recombinant 65 kDa Antigen of *M. Bovis* BCG

The 65 kDa antigen of *M. Bovis* BCG is an example of a recombinant antigen and is currently the focus of much research (67). It is a well characterized and strongly immunogenic protein capable of eliciting antibody and T cell responses in infected patients (47,48,53,66-70). Studies in mice immunized with *M. tuberculosis* have demonstrated that 20% of the mycobacteria-reactive T lymphocytes recognize this molecule (71). Amino acid sequence determination has revealed that the 65 kDa antigen is identical in *M. bovis* and *M. tuberculosis*, and highly homologous to the *Eschericia coli* heat shock protein (HSP) (71). The 65 kDa protein is, therefore, a HSP itself. The human homologue has also been found to be 50% identical with the mycobacterial antigen (72). The HSP are specific proteins produced by cells exposed to a variety of stress stimuli such as elevated temperature, nutrient deficiency, low oxygen pressure, pH alterations, viral infections and reactive oxygen metabolite attack (73). The HSP induce thermotolerance, protect cells from oxidative and other types of injuries, act as molecular chaperones for proteins, and may also play an important role in antigen processing (73).

The 65 kDa antigen has been paradoxically implicated both in the pathogenesis of, and protection against, the development of autoimmunity (47-48,72-77). Lewis rats immunized with heat killed mycobacteria develop a rapidly destructive form of arthritis called adjuvant arthritis. The histological

features of this disease resembles rheumatoid arthritis (78). The autoimmune nature of the disease was suggested by the observation that the disease could be transferred by T lymphocytes (79). These arthritogenic lymphocytes recognize a specific epitope present on the 65 kDa antigen (76). A similar epitope has been found in joint cartilage, suggesting that autoimmune arthritis may be the result of a cross reaction between microbial antigen and host tissue (80,81). Like adjuvant arthritis rats, patients suffering from rheumatoid arthritis demonstrate specific T- lymphocyte reactivity to the *M. tuberculosis* fraction containing the cross-reactive epitope (82).

On the other hand, vaccination with the 65 kDa antigen has been shown to induce resistance to the development of adjuvant arthritis in Lewis rats (76). Resistance was also shown to be dependent on T cell clones able to recognize the same epitope, as that recognized by arthritogenic clones, on the 65 kDa antigen (76). Similarly, streptococcal cell wall-induced arthritis (a T cell dependent chronic, erosive polyarthritis) can be prevented by pretreatment of rats with the 65 kDa antigen (77).

Additional evidence that the 65 kDa antigen may be involved in destructive immune responses has recently come to light (47,48). Recombinant 65 kDa stimulated effector cells were shown to efficiently lyse autologous monocytes in the absence of antigen (47,48). More recent studies have provided a potential mechanism for this phenomenon (74). Bone marrow macrophages "stressed" by exposure to IFN-Ǒ, have been shown to express endogenous proteins bearing cross reactive epitopes to the recombinant 65 kDa antigen (74). These self epitopes are thus capable of sensitizing macrophages for lysis by autologous recombinant 65 kDa primed effector CTL.

The 65 kDa antigen has also been considered a potential candidate for a subunit vaccine (47). Doubt about its widespread application in this regard arose when a study showed that only 20% of 47 BCG responsive individuals (21 healthy contacts and 26 patients with Leprosy) demonstrated significant responses to the 65 kDa antigen (47). However, the study described above (and most other studies involving the 65 kDa antigen) have assessed immune responses to this antigen at the clonal level and in peripheral blood (53,66-70). Responses by cells obtained from the site of the pathology are likely to be more relevant in this regard.

1.2.6.2. The 36 kDa and 65 kDa proteins of *M. Leprae*

These antigens have been purified by either SDS-PAGE or affinity chromatography and screened with a battery of T cell clones and antibodies (66). The 36 kDa antigen was selected for study since it is one of the few antigens which contains a B cell epitope specific for *M. Leprae*. It has in fact already been used in a serodiagnostic test (83). *M. Leprae* specific epitopes have also been defined by T cell clones on both antigens. Interestingly, the antigens isolated from the bacilli contain more epitopes than the corresponding recombinant proteins (66). The most likely explanation for this phenomenon is that additional components are associated with the protein antigens even under the denaturing conditions in SDS-PAGE (66).

The 65 kDa protein of *M. Leprae* has been extensively characterized and 24 T cell epitopes have been documented on this protein to date (84). All have been shown to be major histocompatibility complex (MHC) class II restricted (84). Almost all are HLA DR restricted (84). Thus immunity to the 65 kDa protein is under strict class II immune response gene control. Furthermore, a correlation between specific

peptide, MHC class II and specific T cell receptor has been demonstrated (84). These correlations were noted to be specific to individuals (84). These observations have important implications for vaccine development. Thus future vaccines will have to contain a peptide recognized by most individuals or possibly more than one peptide to account for individual differences in antigen processing.

1.2.7. GAMMA-DELTA T CELLS

Although most T cells express the alpha-beta receptor heterodimer, a small percentage of thymocytes and peripheral blood lymphocytes have been shown to express an alternative receptor known as the gamma-delta receptor (85). While the functions of T lymphocytes expressing the conventional alpha-beta receptor have been extensively studied, gamma-delta T cells represent a subset with an as yet uncertain role. In the last few years some clues have emerged pertaining to a possible function for these cells. The first of these was the observation that gamma-delta cells show non-specific cytotoxicity (86). The second was that gamma-delta T cells show site specificity. Non circulating lymphocytes found within murine skin and gut are largely composed of gamma-delta cells (87,88). In addition the gamma-delta receptors born by these cells appear to be highly restricted despite significant germline diversity. (87,88). Thirdly, gamma-delta T cells mature in the fetal thymus prior to alpha-beta cells (89). Thus the implication arising from these observations is that these cells may represent a first line of defence and are therefore found at sites where foreign invaders would most likely intrude first (90). Consistent with these notions was the observation that gamma-delta T cells were greatly increased in the draining nodes of mice after primary immunization with *Mycobacterium tuberculosis* (91). Analysis of cell cycle, interleukin-2 receptor expression and of interleukin-2 responsiveness

showed that a large proportion of these cells were activated *in-vivo* (91). Significantly, the gamma-delta T cell population showed virtually no expansion after secondary immunization (91).

Experiments with gamma-delta hybridomas isolated from an antigen-unselected population of neonatal thymocytes have resulted in intriguing observations further clarifying the antigen reactivity of these cells (90). More than a third of the hybridomas were found to spontaneously produce interleukin-2 (90). Of several antigens tested, the hybrids only responded to mycobacterial antigen (90). Strikingly, the PPD responsive hybrids were precisely those that produce interleukin-2 spontaneously (90). Further testing of the PPD responsive hybrids showed that most of them responded to the recombinant 65 kDa protein of *M. Bovis* BCG (90). These data therefore imply that at least a significant fraction of gamma-delta T cells are specialized to recognize epitopes on a class of phylogenetically conserved stress proteins or HSP which have previously been implicated as immunodominant mycobacterial antigens (92).

1.2.8. IMMUNOTHERAPY

Recent evidence has suggested that the immune reactions responsible for protection in tuberculosis are distinct from those contributing to the pathogenesis of the disease (9,13). Furthermore, pre-immunization of mice with mycobacterial antigen, so that delayed hypersensitivity is enhanced, has been shown to either greatly increase susceptibility of the skin challenge site to TNF or to lead to a complete loss of such sensitivity (13). The nature of the response was determined by the nature of the antigen. Thus mice pre-immunized with subcutaneous sonicated *M. Vaccae* in Complete Freund's adjuvant (CFA) rendered delayed hypersensitivity sites TNF insensitive while pre-

immunization with either *M. Vaccae* or CFA alone resulted in extreme TNF sensitivity (13). These observations have been extended to patients and preliminary data are encouraging (93-96). Subcutaneous injections of 10^9 irradiation-killed *M. vaccae* into tuberculosis and leprosy patients resulted in restoration of immune recognition of common mycobacterial antigens and a reduction in the necrotic component of the tuberculin test without causing harmful side effects (94). Significant weight gain has also been noted in treated patients when compared to those not receiving immunotherapy (94). More recently, it has been shown that patients vaccinated with *M. Vaccae*, which had been subjected to various modifications, showed enhanced and probably beneficial alterations in immunological activity around pulmonary lesions resulting in closure of cavities (95). This study also indicated that the immunotherapeutic agent is open to potentially beneficial modifications. Thus much more work is required and elucidation of the mechanism of immunotherapy at the cellular and molecular level might lead to a considerable refinement of what is, at present, a somewhat empirical approach (96).

1.3. PERSISTING IMMUNE PHENOMENA IN TUBERCULOSIS

At least three abnormalities that accompany tuberculosis are known to be inexplicably persistent. Necrotizing skin-test responsiveness can persist for years following tuberculosis (97). Also, a change in the pattern of glycosylation of the Fc portion of IgG (agalactosyl IgG) has been found to remain grossly abnormal in most tuberculosis patients long after clinical cure (98). Recently, alveolar macrophages obtained by bronchoalveolar lavage, from a patient with tuberculosis, were shown to actively synthesize $1,25(OH)_2D_3$ ten months after successful antituberculous therapy (99). The continued capacity to produce $1,25(OH)_2D_3$ was associated with a persistent lymphocytic alveolitis, in spite of the patient's

apparent clinical recovery (99). No evidence of *M. tuberculosis* could be found in direct smears and cultures of sputum, and bronchial aspirates were also negative (99). These authors considered that the persistent alveolitis was due to an ongoing inflammatory and immune response in the absence of *M. tuberculosis* (99). Others have, however, not ruled out the possibility that persistent mycobacterial components were causing the alveolitis (98).

1.4. LONG TERM ANTIGEN PERSISTENCE

1.4.1. ANIMAL STUDIES

The evidence indicating that antigen can persist for months or years in the spleens and draining lymph nodes of immunized animals is persuasive and has been extensively reviewed (100). Studies employing even readily degradable radio-iodinated protein antigens such as human serum albumin have confirmed long term antigen retention (100). These studies have also precisely localized long term retained antigen to the processes of follicular dendritic cells found in the draining lymph nodes of immunized mice (100). It is thought that persisting antigen at this site plays an important role in the maintenance and regulation of the humoral immune response (101).

1.4.2. CLINICAL EVIDENCE OF LONG TERM ANTIGEN PERSISTENCE

Persisting antigen has been implicated in the pathogenesis of a number of chronic diseases (102-104).

Recently, putative persisting Yersinia antigen was demonstrated (by immunofluorescence and western blotting) in synovial-fluid cells obtained from patients with reactive arthritis (102). Extensive bacterial cultures were negative.

In one case, the antigen was detected 17 years after initial infection with Yersinia (102). However, the possibility that the specific anti-Yersinia antibodies used to detect the Yersinia antigen, was cross-reacting with a self epitope cannot be ruled out (102).

Lyme borreliosis is a multisystemic infectious disease caused by the spirochete Borrelia burgdorferi. Antigen persistence is thought to be the most likely pathogenetic mechanism of Lyme disease (104). Limiting dilution analysis of peripheral blood lymphocytes from patients with chronic Lyme arthritis, have shown both an increased precursor frequency of cells reactive with Borrelia antigen, as well as a high proportion (23-100%) of *in-vivo* activated cells (104). Surprisingly, limiting dilution analysis of these cells 6 to 16 months after apparent clinical cure documented identical frequencies of *in-vivo* activated cells (104). Since the patients were asymptomatic, these observations may be the result of persisting antigen. Alternatively, anti-idiotypic interactions via antigen receptors may be responsible for the maintenance of the activated Borrelia-reactive T cells (104).

1.5. SPONTANEOUS LYMPHOCYTE PROLIFERATION

Spontaneous lymphocyte proliferation has not, prior to this work, been described in tuberculosis. The number of other diseases associated with this phenomenon, however, is mounting. It has been observed in Sarcoidosis, Rheumatoid arthritis and a number of viral infections (105-117). The phenomenon has also been observed in autoimmune MRL/Mp-lpr/lpr mice (118-120).

1.5.1. SARCOIDOSIS.

In this condition alveolar T lymphocytes obtained from patients with high intensity alveolitis were observed to proliferate and produce interleukin-2 (IL-2) spontaneously (105,106). The proliferation was observed in both fresh unseparated mononuclear cells and purified T lymphocytes (105,106) cultured for 24 hours. These findings were restricted to alveolar lymphocytes and could not be demonstrated in the peripheral blood (105,106). The proliferation could be abrogated with steroid therapy and was thought to account for the increased number of cells found at the site of the pathology in this disease (107,108). In these earlier studies the initiating mechanism was not identified (105,106). It was, however, postulated that either all the requisite signals for T cell activation were present in the alveolar structures in patients with sarcoidosis and high intensity alveolitis, or that the effector cell population in the lungs of these patients could release IL-2 without all the requisite signals. Evidence supporting the former possibility appeared in a later study (109). In that study alveolar macrophages were shown to possess an enhanced capacity to present antigen (109). This finding hinted at the possibility that persisting foreign antigen may be responsible for the initiation of the phenomenon.

1.5.2. RHEUMATOID ARTHRITIS.

The phenomenon in this disease shares similarities with its counterpart in Sarcoidosis, namely IL-2 dependent spontaneous proliferation by short term cultured lymphocytes obtained from the site of the pathology (110). Peripheral blood lymphocytes, however, demonstrated enhanced proliferative responses to exogenous IL-2 in spite of having no significant increase in the classic activation markers such as HLA DR, Tac and Transferrin (110). The spontaneous proliferation was shown to be largely dependent on dendritic

cells (110). It was therefore concluded that the phenomenon was the result of an autologous mixed lymphocyte reaction (110). However, the presence of persisting foreign antigen in the dendritic cells was not excluded.

1.5.3. VIRAL INFECTIONS.

Spontaneous proliferation in the peripheral blood has been observed in Measles, Mumps and Rubella (111,112). It has also been observed in the early phases of *in-vitro* infection with human T cell leukemia virus types I and II (HTLV-I and -II) (121). The phenomenon has been most intensely studied in measles and HTLV infected patients (111,112,121).

1.5.3.1. Measles

Most of the serious complications of measles are due to secondary bacterial and viral infections causing pneumonia and diarrhoea, or to post-measles encephalomyelitis, an autoimmune demyelinating disease (113-116). Both types of complications, infectious and autoimmune, are thought to be related to the immunologic abnormalities that accompany acute measles (111,117). Peripheral blood lymphocyte activation and spontaneous proliferation is one such abnormality (111,112). In this disease, spontaneous proliferation was noted in the peripheral blood to appear coincident with the onset of the rash and to persist for approximately 10 days (111,112). It was, therefore, thought that the spontaneous proliferation represented cells which were released into the peripheral blood from infected tissue, the primary site where cellular proliferation is induced by viral antigen and interleukin-2 (111). This release was thought to occur during vigorous phases of cellular expansion (111).

1.5.3.2. Human T cell leukemia virus types I and II.

It is well recognized that HTLV-I and HTLV-II infection *in-vitro* induces an early polyclonal T cell proliferation *in-vitro* that is supplanted by a monoclonal, growth-factor independent outgrowth of leukemic cells (121). A viral gene and protein has been observed to activate interleukin-2 genes, interleukin-2 receptor genes, and class II histocompatibility genes (121). The early spontaneous proliferation is thus thought to involve virus gene induced autocrine proliferation (121). The subsequent events that result in the emergence of a clonal growth factor independent leukemic T cell population remain undefined (121).

1.5.4. Spontaneous Lymphocyte Proliferation in MRL/MP-LPR/LPR Mice.

MRL/Mp-lpr/lpr mice develop an age dependent progressive lymphoproliferative disease, which is characterized by the massive accumulation of an unusual subset of lymphocytes in their lymphoid organs (118,119). These mice develop a rapid onset systemic lupus erythematosis-like syndrome characterized by autoantibody production (118,119). Lymph node T cells from these mice but not from congenic MRL/Mp-+/+ have been shown to proliferate spontaneously when cultured *in-vitro* for 5 to 7 days (118). In contrast with the other conditions discussed above (and similar to our observations in a subset of patients with tuberculosis), freshly isolated lymphocytes did not proliferate spontaneously (118). Spontaneous proliferation was only seen after 5 to 7 days of *in-vitro* culture (118). The length of *in-vitro* culture and initial culture density was shown to be critical, indicating that cell to cell interaction was essential for the initiation of the proliferation (118). The proliferation could be blocked by both anti-T4 and anti-IL-2-receptor antibodies (118). This confirmed the phenomenon to be IL-2 dependent and, in addition, indicated that the

proliferating cells were $CD4^+$. An intriguing observation made in these studies was that the spontaneous proliferation and IL-2 production could be detected in young MRL/Mp-lpr/lpr mice at a time when lymphoproliferation and autoimmunity were not obvious (118). The relationship between the spontaneous proliferation and the development of autoimmunity was not established in these studies. The cells involved in the spontaneous proliferation were shown to be $CD4^+$ and therefore cannot be the abnormal T cells shown to accumulate in lpr/lpr mice since it is known that these abnormal T cells lack the CD4 marker (120). However, these authors speculate that the spontaneously proliferating lymphocytes may produce lymphokines that accelerate the growth of the abnormal T cells or enhance the polyclonal activation of B cells (resulting in autoantibody production) (118).

1.6. CONCLUSION

It is clear from the above review that cellular immune mechanisms play a prominent role after infection with *M. Tuberculosis*. This thesis therefore examines several aspects of the cellular immune response in tuberculosis in an attempt to further define protective immune responses.

As discussed above, the development of caseation within granulomata has been shown to be associated with an abrupt end to mycobacterial multiplication. *In-vitro* evidence suggests that cell mediated cytotoxicity and/or cytokine release may result in caseation. *In-vivo* evidence for cell mediated cytotoxicity against macrophages infected with *M. Tuberculosis* is however lacking. Thus particular attention was paid to cell mediated cytolysis in patients with active tuberculosis.

Proliferation and cytotoxicity to the recombinant 65 kDa antigen of *M. Bovis* BCG was also assessed in these patients because this antigen has been shown to be immunodominant in mice, is a HSP, and has been considered as a potential candidate for a vaccine.

CHAPTER 2

DESCRIPTION OF A NOVEL IMMUNE PHENOMENON IN TUBERCULOSIS: PERSISTENT IN-VITRO SPONTANEOUS PERIPHERAL BLOOD LYMPHOCYTE PROLIFERATION

2.1. ABSTRACT

Spontaneous lymphocyte proliferation was noted *in-vitro*, in lymphocytes obtained from the peripheral blood of a subset of patients with tuberculosis. The proliferation persisted in some cases and was noted in one case four years after apparent clinical and radiological cure. Proliferation was assessed by ^{3}[H]thymidine incorporation. Receptor studies, including immunostaining and blocking with anti-interleukin-2-receptor antibody, indicated that the proliferation was mediated by the major T-cell lymphokine interleukin-2. Several levels of evidence implicated mycobacterial antigenic components in the initiation of the proliferation. Firstly,the kinetics of the proliferation resembled that of antigen-driven T cell proliferation. Secondly, the proliferation had a strict requirement for the presence of accessory cells, since: i) adherent cell depletion abrogated the phenomenon, ii) adherent cell supernatants obtained from patients with high spontaneous proliferation caused significant stimulation only when added to unseparated control cells obtained from a known PPD responder, and not when added to purified T cells obtained from the same control PPD responder. Thirdly, adherent cell supernatants obtained from control tuberculosis patients lacked stimulatory activity despite having detectable levels of thymocyte co-mitogenic activity. Taken together, the data suggests continuous secretion of antigenic mycobacterial components capable of stimulating T-cell proliferation in a subset of patients following infection with *M. tuberculosis*.

2.2. INTRODUCTION

Lymphocytes plated in conventional *in-vitro* cultures do not usually proliferate unless stimulated with exogenous antigen. Spontaneous lymphocyte proliferation has however been observed in a number of disease states. These include sarcoidosis, rheumatoid arthritis and a number of viral infections (105,106,110-112). It has also been observed in an animal model of systemic lupus erythematosis (118,119).

In the course of routinely assessing cellular immune responses in patients infected with *M. tuberculosis*, a subset of patients were observed to have spontaneous peripheral blood lymphocyte proliferation. The phenomenon was noted to persist in a number of these patients.

At least three other abnormalities associated with tuberculosis have been shown to persist. Necrotizing skin test responsiveness may persist for years following tuberculosis (97). A change in the pattern of glycosylation of the F_C portion of IgG has been found to remain abnormal in most TB patients long after clinical cure (98). More recently, the serum levels of $1,25(OH)_2D_3$ in a patient with TB was shown to peak nine months after the start of treatment, when the patient was clinically well (99). This patient still had an active alveolitis at the time and lavage cells were found to be synthesizing active metabolites (99).

The mechanisms underlying these persisting phenomena are not fully understood. In this study, evidence suggesting that spontaneous lymphocyte proliferation in tuberculosis may be due to persisting mycobacterial components is provided.

2.3. MATERIAL AND METHODS

2.3.1. Study population: Tuberculosis

A total of 46 patients were studied. Active tuberculosis (TB) was diagnosed in 45 patients by the presence of acid fast bacilli in the sputum or biopsy specimen. Thirty four had pulmonary TB, four had nodal TB, three patients had disseminated disease, three had tuberculous pleuritis, and one had miliary TB. The remaining case had non-specific symptoms without evidence of overt disease. This patient had sputum positive pulmonary TB four years prior to inclusion in the study. Two patients had received treatment at the time they were first studied and one had intermittent courses prior to inclusion in the study.

2.3.2. Study population: normals

The 22 controls were all young laboratory staff and medical students.

2.3.3. Isolation and separation of peripheral blood mononuclear cells

Heparinised (thromboloquine, 250 units/15 ml blood, Organon Teknika 80701596, Boxtel, Holland) peripheral blood was separated by Ficoll-Hypaque density gradient centrifugation essentially as described (122). The blood was spun at 350G for 12 minutes. The buffy coat was removed, diluted with an equal volume of phosphate buffered saline (PBS) and overlaid on 3 ml of Ficoll-Hypaque in sterile plastic tubes (Falcon 2025, Becton Dickinson, California, U.S.A.). The tubes were spun at 400G for 30 minutes and the interface was removed, diluted with PBS and the cells were pelleted by centrifugation. The peripheral blood mononuclear cells (PBMC) thus obtained were washed 2 more times with PBS and utilized as unseparated PBMC or further fractionated as described below.

2.3.4. Spontaneous proliferation of PBMC

Freshly isolated unstimulated PBMC (1 x 10^5) were added to wells of 96 well round-bottomed sterile tissue culture plates (Flow Laboratories) in 0.2 ml of RPMI 1640 tissue culture media (Flow Laboratories, Irvine, Scotland) containing 10% pooled AB human serum, 100 u/ml penicillin and 100 mg/ml streptomycin (Gibco, Grand Island, N.Y.) hereafter referred to as complete medium. Control cells were in all cases plated at the same time in the same complete media. Autologous serum was used in place of pooled human serum in some cultures and some cultures also contained varying concentrations of either autologous or pooled human serum ranging from 10% to 30%. A prostaglandin inhibitor (indomethacin, Sigma 17378, Sigma-Aldrich Corporation, St. Louis, Missouri) was added to some cultures at concentrations ranging from 2% to 0.25%. The cultures were incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Tritiated thymidine (2 µCi/well, specific activity 185 Mega BQ, Amersham 120, Amersham Laboratories, Buckinghamshire, England) was added to cultures on either day 3, day 6 or day 9 for an additional 16-18 hours. The cells were harvested using an automated harvester (Titertek 630), and radioactivity was measured using a liquid scintillation counter (Packard, Tricarb 4640). All assays were performed in triplicate. Spontaneous proliferation was considered significant if > 3000 cpm (approximately the sum of the mean and 3 standard deviations of 22 control subjects).

2.3.5. Quantification of IL-2 receptor and HLA-DR expression

The IL-2 receptor was identified by using 100µl of the monoclonal antibody IL-2R1 (10 µg/ml, Coulter, Hialeah, Florida). L-243 ascites (124) was used to identify HLA-DR. Briefly, the relevant antibody was added to 1 x 10^6 cells

resuspended in 100µl at 4 ^{o}C for 45 minutes. The cells were washed 3 times with PBS containing 2.5% FCS and 0.02% sodium azide and 100µl of the appropriate dilution of a fluorescein iso-thiocyanate conjugated (FITC) $F(ab)'_2$ goat anti-mouse (Cappel, Cooper Biomedical) immunoglobulin reagent was added to the cells at 4 ^{o}C for an additional 30 minutes. The cells were washed 3 times and examined by indirect fluorescence microscopy. At least 200 cells were counted for each sample.

2.3.6. Effect of anti-IL-2 receptor antibody on spontaneous proliferation

Anti-IL-2-receptor antibody (anti-Tac, a kind gift from Dr Waldman, NIH, Bethesda) was added to cultures at 5 and 10 µg/ml and the effect on proliferation was assessed as described above.

2.3.7. Effect of adherent cell depletion

PBMC were adhered to plastic petri dishes (Falcon,Becton Dickinson) for 45 minutes. The non adherent cells (NAC) were gently decanted. 1 x 10^5 NAC were plated in 0.2ml complete medium. Spontaneous lymphocyte proliferation was quantitated as described above.

2.3.8. Identification of adherent cells

Adherent cells were recovered following treatment with cold 0.08% EDTA-PBS (BDH Chemicals Ltd, Poole, England) and incubated for a further 15 minutes at 37^{o}C. 1 x 10^6 recovered cells were stained with the monocyte/macrophage marking monoclonal antibody MY4 (Coulter, Hialeah, Florida) followed by FITC and percent fluorescence was determined as described above.

2.3.9. Effect of adding non-T (E^-) to purified T cells (E^+)

PBMC were rosetted using sheep red blood cells (SRBC) (123). SRBC-absorbed pooled AB human serum was prepared in advance. The human serum (HS) was absorbed, at a ratio of 2:1, to washed SRBC for 1 hour at 37°C followed by an overnight incubation at 4°C. The absorbed HS was filtered and stored at -20°C until required. On the day of cell separation, fresh SRBC were washed 3 times and made up to 3% in 40% SRBC absorbed HS-PBS in a total volume of 10 ml. Unseparated PBMC were reconstituted at 5 x 10^6/ml in 10 ml of 40% HS-PBS. Equal volumes of cells in 40% HS-PBS and 3% SRBC-40% absorbed HS-PBS were mixed and underlaid with 15 ml Ficoll-Hypaque in 50 ml sterile plastic tubes (Falcon 2025, Becton Dickinson, California, U.S.A.). The tubes were incubated for 1 hour at 4^0C and then spun at 800G for 30 minutes. The non-T cells contained in the interface were removed, washed 3 times in 5% HS-PBS and resuspended in complete medium. The pellet containing the T cells bound to SRBC was resuspended in NH_4Cl (0.77%) and incubated for approximately 5 minutes at 37°C until all SRBC were lysed when the reaction was stopped with cold PBS. The cells thus obtained were washed 3 times with 5%HS-PBS and resuspended in complete medium. Each cell subset was plated in triplicate at 1 x 10^5 per well. To determine which cells were proliferating and what the requirements for activation were, non-T cells (5 x 10^4) were added back to 5 x 10^4 purified T cells and proliferation determined as described above. The non-T cells were irradiated (1000 rads) in some experiments, prior to addition to culture.

2.3.10. Analysis of adherent cell supernatants

Supernatants of adherent cells from patients with and without high spontaneous proliferation (and also control subjects) was obtained by adhering 2 x 10^6 unstimulated PBMC to sterile plastic petri dishes for 45 minutes. The NAC were decanted and the adherent cells were gently washed three

times. 1 ml of complete medium was added to the adherent cells and removed 24 hours later to be frozen at -20 ^{o}c until tested in the thymocyte co-mitogenesis assay as previously described (125). Briefly, the thymus gland of a 6 week old BALB/c mouse was aseptically removed and teased apart. The cells thus obtained were washed 3 times and resuspended in RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (Gibco, 011-6290, Gibco Middlesex, England) , 100 u/ml penicillin and 100 mg/ml streptomycin (Gibco, Grand Island, N.Y.). Thymocyte density was adjusted and 1 x 10^{6} cells were placed in each well of a round-bottom microtitre plate (Flow Laboratories, Irvine, Scotland). In addition to the cells each well contained a submitogenic dose of phytohaemagglutinin (PHA) (HA 16, Welcome Diagnostics, Temple Hill, Dartford, England, a 1:400 dilution was found to be submitogenic in earlier assays) and the test supernatant or complete medium in a final volume of 200 µl. Supernatants obtained from control adherent cells cultured in the presence of *E. coli* Lipopolysaccharide (LPS, Difco) served as positive controls. The cultures were incubated for 72 hours and [^{3}H]thymidine was added during the last 18 hours. The cells were harvested and [^{3}H]thymidine incorporation was determined as described above. Results were expressed as stimulation indeces. The stimulation index was calculated as follows: mean cpm in wells containing PHA and test supernatant/mean cpm in wells containing PHA and complete medium. A stimulation index of greater than 3 was considered a positive result.

The capacity of these supernatants to stimulate proliferation was also analyzed by adding them to NAC obtained from patients with and without spontaneous lymphocyte proliferation. In addition, the supernatants were added to control cells obtained from a known normal PPD responder, both in the presence and absence of antigen

presenting cells (APC). APC were removed from the control cells by twice rosetting with SRBC followed by two cycles of plastic adherence. Proliferation was quantitated on day 6 as described above.

2.3.11. Statistical analysis

Confidence intervals were calculated as described (126). Analysis of variance by comparison of means was used to calculate P values.

2.4. RESULTS

2.4.1. Spontaneous lymphocyte proliferation in TB

In-vitro cultured unstimulated PBMC from 6 of 46 patients showed high spontaneous lymphocyte proliferation when compared to the normal controls (Fig.2.1). The proliferation was statistically significant in all 6 cases ($P < 0.01$). None of the cells obtained from normal individuals manifested the phenomenon and their proliferation was similar to that of the remaining 40 patients with tuberculosis (Fig.2.1) (Mean difference 284, 95% confidence interval (CI) -20 to 588; $t = 2.0$, degrees of freedom (df) = 60, $P > 0.05$). Plating on different areas of the same plate and also simultaneously on separate plates reproduced the phenomenon consistently. Parallel cultures comparing pooled human serum and autologous serum yielded identical results (Table 2.1). The proliferation was also not influenced by the prostaglandin inhibitor indomethacin (Table 2.2).

2.4.2. Spontaneous proliferation over time

In one case (Fig.2.2.1), the phenomenon persisted for 13 months and possibly longer, since the patient was lost to

Fig. 2.1. Spontaneous lymphocyte proliferation in patients with TB compared to normals. Unstimulated PBMC, 10^5 ,from 46 TB patients and 22 controls were cultured for 6 days and pulsed with [^{3}H]thymidine for the last 16-18 hours.

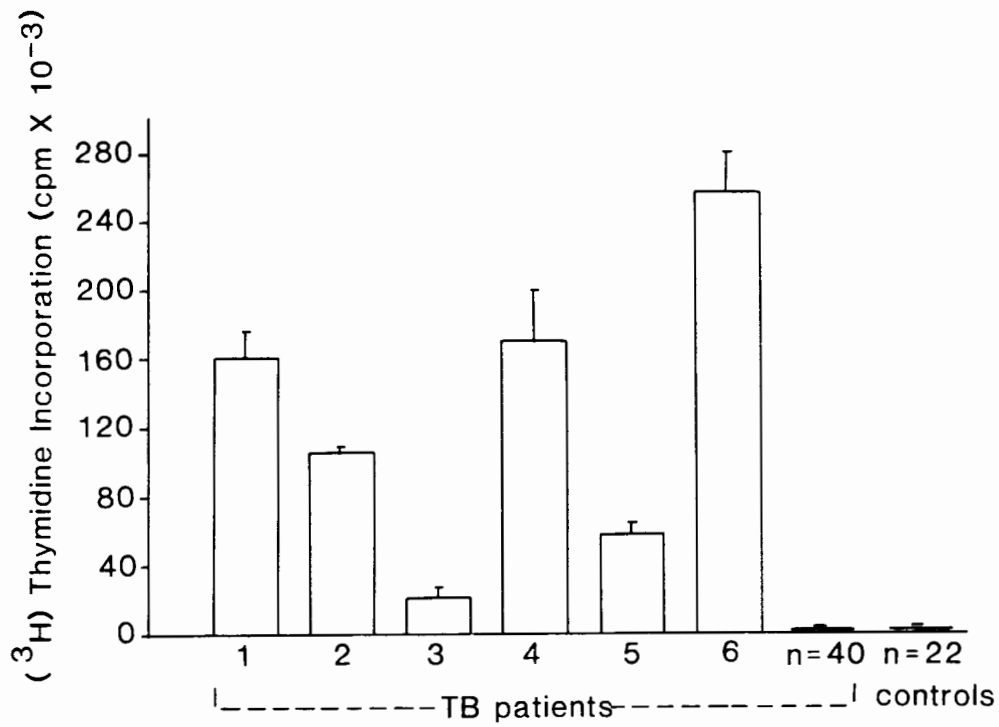


Table 2.1. Spontaneous lymphocyte proliferation in autologous serum compared to pooled human serum.

PATIENT	PERCENT HS*	POOLED HS	AUTOLOGOUS HS
1	10%	72936 ± 19765	68737 ± 35567
	20%	56071 ± 15630	47298 ± 15114
	30%	59292 ± 2270	48789 ± 4315
2	10%	67809 + 243	110011 ± 25822
	20%	100909 ± 10568	89698 ± 545

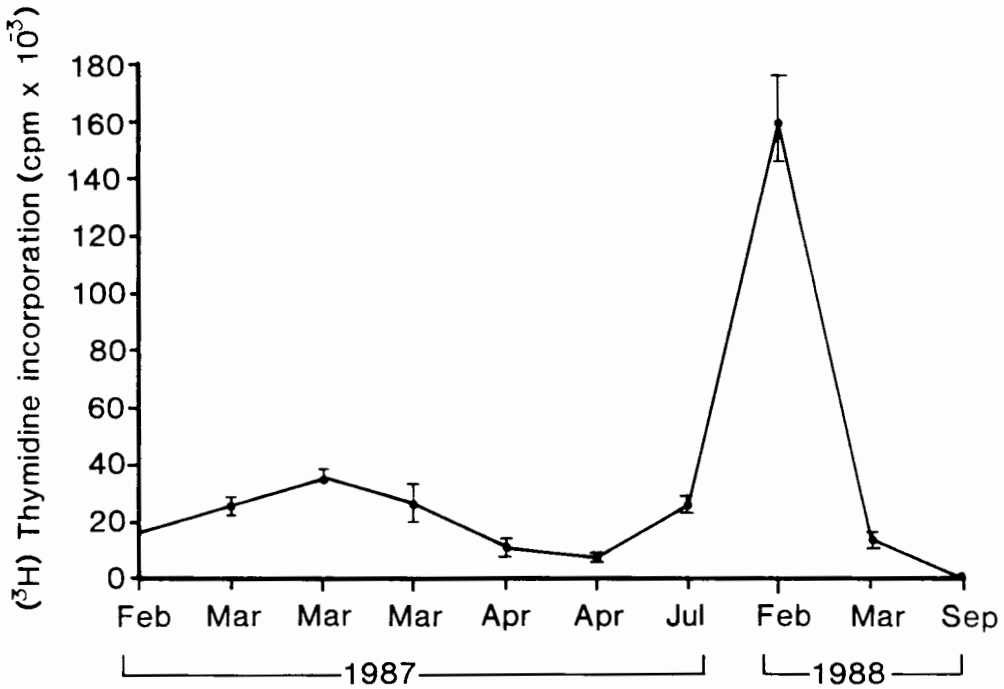
Unstimulated PBMC were cultured for 6 days in RPMI supplemented with varying concentrations of either pooled human or autologous serum. Each culture was pulsed with [^{3}H]thymidine for the last 16-18 hours. Values are mean cpm ± SD. Data represent the means of triplicate determinations.
*HS, human serum

Table 2.2. Effect of the prostaglandin inhibitor indomethacin on spontaneous lymphocyte proliferation.

PATIENT	% INDOMETHACIN	MEAN CPM ± SD
1	2%	14344 ± 1684
	1%	13728 ± 2988
	0.5%	13232 ± 2100
	0.25%	9400 ± 557
	0%	13332 ± 1357
2	2%	38935 ± 16012
	1%	46976 ± 11419
	0.5%	49079 ± 6629
	0.25%	46686 ± 17980
	0%	46059 ± 4785

Unstimulated PBMC were cultured for 6 days in complete medium. A prostaglandin inhibitor was added at the doses shown. Each culture was pulsed with [^{3}H]thymidine for the last 16-18 hours. Values are mean cpm ± SD. Data represent the means of triplicate determinations.

Fig. 2.2.1. Spontaneous lymphocyte proliferation over time (Patient 1). Unstimulated PBMC were cultured for 6 days and pulsed with [^{3}H]thymidine for the last 16-18 hours. This was repeated at the time intervals indicated.



follow up until the eighteenth month when spontaneous lymphocyte proliferation was no longer present. In this patient the abnormality persisted at least 4 months after complete clinical and radiological resolution of disease. The proliferation persisted for the duration of the follow up period (4 months) in a second case (Fig.2.2.2). No decrease in the level of proliferation was detected in this patient in spite of gradual clinical improvement. The proliferation persisted for 14 months in a third case without any clinical or radiological evidence of disease activity (Fig.2.2.3) and was transient in two cases. The sixth patient was lost to follow up and no longitudinal data is available.

2.4.3. IL-2 receptor expression and blocking

The spontaneously proliferating cells expressed IL-2 receptors on day 6 (Table 2.3). IL-2 receptors were neither detected on fresh PBMC obtained from these patients (Table 2.3) nor on day 6 control cells. Anti-IL-2 receptor antibody (anti-Tac) blocked the proliferation in a dose-dependent manner (Fig.2.3). In repeated experiments, the percentage reduction in proliferation ranged from 21 to 75% at 5μg/ml anti-Tac and from 30 to 87% at 10μg/ml anti-Tac.

2.4.4. HLA-DR expression

Expression of HLA-DR antigen was not significantly increased in fresh PBMC obtained from patients with spontaneous lymphocyte proliferation (Table 2.4). However, an increase in HLA-DR was observed, in cells obtained from patients with spontaneous proliferation after 6 days of unstimulated culture (Table 2.4). Similar changes were not observed in normal control cells and cells obtained from patients without spontaneous proliferation (Table 2.4).

Fig. 2.2.2. Spontaneous lymphocyte proliferation over time (Patient 2). Unstimulated PBMC were cultured for 6 days and pulsed with [^{3}H]thymidine for the last 16-18 hours. This was repeated at the time intervals indicated.

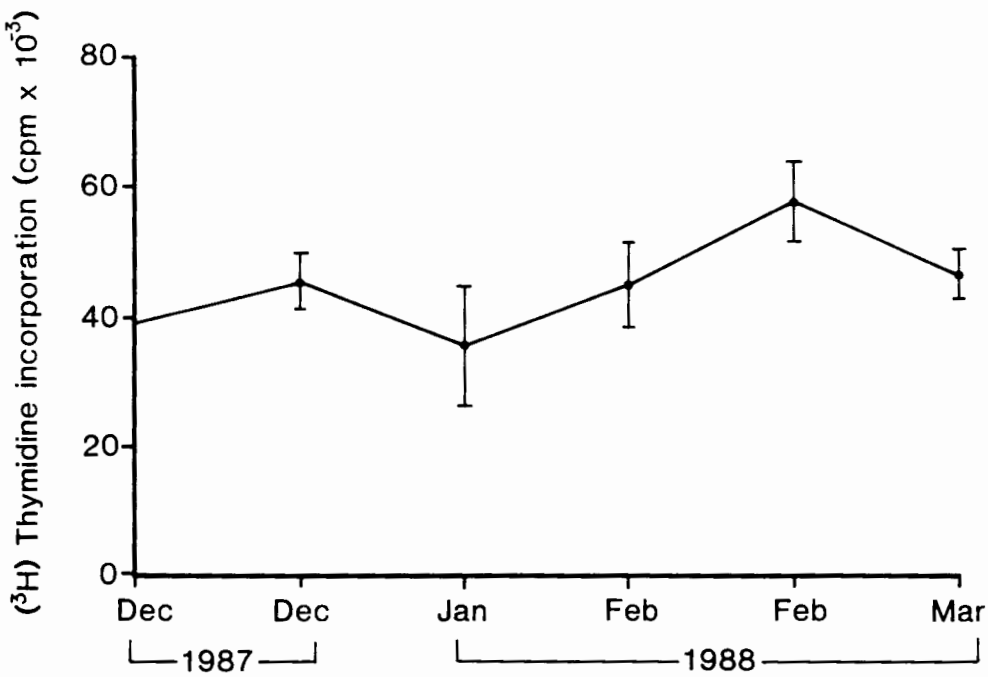


Fig. 2.2.3. Spontaneous lymphocyte proliferation over time (Patient 3). Unstimulated PBMC were cultured for 6 days and pulsed with [^{3}H]thymidine for the last 16-18 hours. This was repeated at the time intervals indicated.

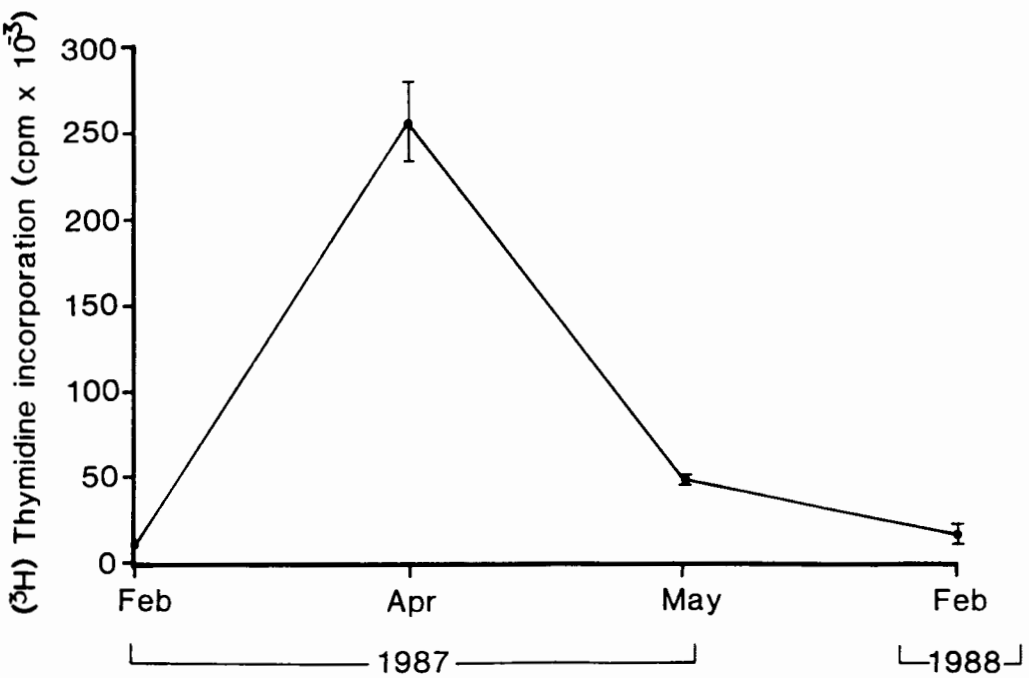


Table 2.3. Percent IL-2 receptor expression by PBMC obtained from patients with spontaneous lymphocyte proliferation.

	% IL-2 RECEPTORS	
PATIENT	DAY 0	DAY 6
1	0	20
2	0	11
3	0	10

Unstimulated PBMC were cultured for 6 days and then stained for IL-2 receptor expression using indirect fluorescence. Data represent percentage of cells staining positive by indirect fluorescence microscopy.

Fig. 2.3. Blocking of spontaneous lymphocyte proliferation by anti-IL-2 receptor antibody. Anti-IL-2 receptor antibody was added to unstimulated PBMC at 5 and 10 μg/ml and proliferation assessed on day 6 by [^{3}H]thymidine incorporation into DNA.

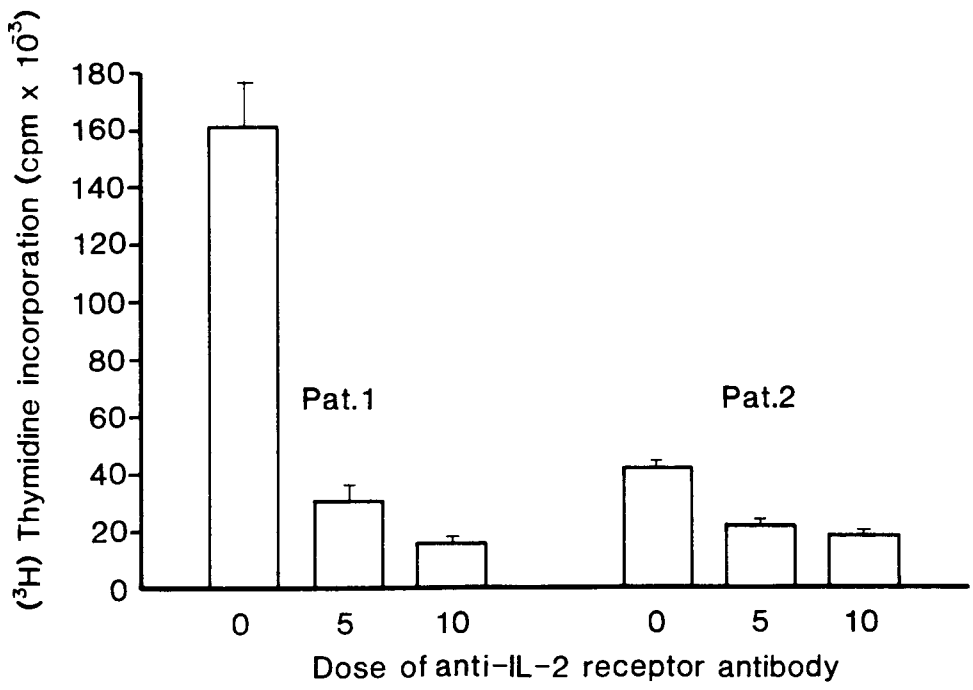


Table 2.4. HLA-DR expression by unstimulated PBMC obtained from TB patients and controls.

	PERCENT OF CELLS POSITIVE:	
EXPERIMENT	DAY 0	DAY 6
1	6	18
2	11	25
3	2	10
4	14	3
5	6	0
6	21	0
7	7	0
8	4	0

Results are expressed as percent positively staining cells as determined by indirect fluorescence microscopy.
Experiments 1-3 were performed on cells obtained from patients with spontaneous lymphocyte proliferation.
Experiments 4-6 were performed on cells obtained from patients without spontaneous lymphocyte proliferation.
Experiments 7 and 8 were performed on cells obtained from control subjects.

2.4.5. Kinetics of spontaneous proliferation

The kinetics of the proliferation resembled that of antigen driven proliferation with peak proliferation on day 6 of culture (Table 2.5). All cases demonstrated the same pattern of kinetics.

2.4.6. Effect of removing adherent cells

Depleting the PBMC of adherent cells by plastic adherence almost completely abrogated the proliferation in 4 of 5 patients tested (Table 2.6). By this relatively crude technique spontaneous proliferation could not be abrogated in only one patient (patient 5, Table 2.6).

2.4.7. Identification of adherent cells

The percentage of adherent cells staining positive with the monocyte marker MY4 was 78%, 80% and 82% in 3 experiments respectively. This confirmed that the majority of the adherent cells were monocytes.

2.4.8. Effect of adding back non-T (E^-) cells

Adding back viable (Fig.2.4.1) or irradiated (Fig.2.4.2) non-T cells (E^-) to autologous purified T cells (E^+) reconstituted and augmented the proliferation. Similar treatment of control cells resulted in only low levels of proliferation, as shown in the figures. This confirmed the requirement for T/non-T cell interaction in the initiation of this phenomenon and that the proliferating cell was a T lymphocyte.

2.4.9. Effect of adding adherent cell supernatants to non-adherent cells

Table 2.5. Kinetics of spontaneous lymphocyte proliferation.

PATIENT	DAY 3	DAY 6	DAY 9
1	2240 ± 403	26842 ± 6554	2271 ± 1633
2	18946 ± 294	39102 ± 2890	ND*
3	2363 ± 355	169763 ± 30474	ND
4	23406 ± 1300	106051 ± 2913	ND
5	4810 ± 382	16417 ± 5940	ND
6	2584 ± 1010	121730 ± 18433	34486 ± 1386

Unstimulated PBMC were cultured for 3, 6 and 9 days in complete medium. Each culture was pulsed with [^{3}H]thymidine for the last 16-18 hours. Values are mean cpm ± SD. Data represent the means of triplicate determinations.
*ND, not done.

Table 2.6. Effect of adherent cell depletion.

PATIENT	US PBMC+	NAC
1	161432 ± 15024	518 ± 29
2	106051 ± 2914	451 ± 111
3	48704 ± 1595	1105 ± 577
4	169763 ± 30474	392 ± 305
5	54688 ± 3074	55733 ± 2432

NAC or unseparated PBMC (1 x 10^5/well) were cultured without addition of antigen in 96 well tissue culture plates in complete medium. To obtain NAC, PBMC were incubated on plastic petri dishes for 45 minutes after which NAC were decanted and collected. Values are mean cpm ± SD. Data represent the means of triplicate determinations.
+US PBMC, Unseparated peripheral blood mononuclear cells.

Fig. 2.4.1. Effect of adding back non-T (E^-) cells to purified T (E^+) cells. PBMC were rosetted using SRBC. 10^5/well each of unseparated (US), non-T (N) and purified T cells (T) were cultured in parallel. 5 x 10^4 non-T cells were added back to 5 x 10^4 purified T cells.

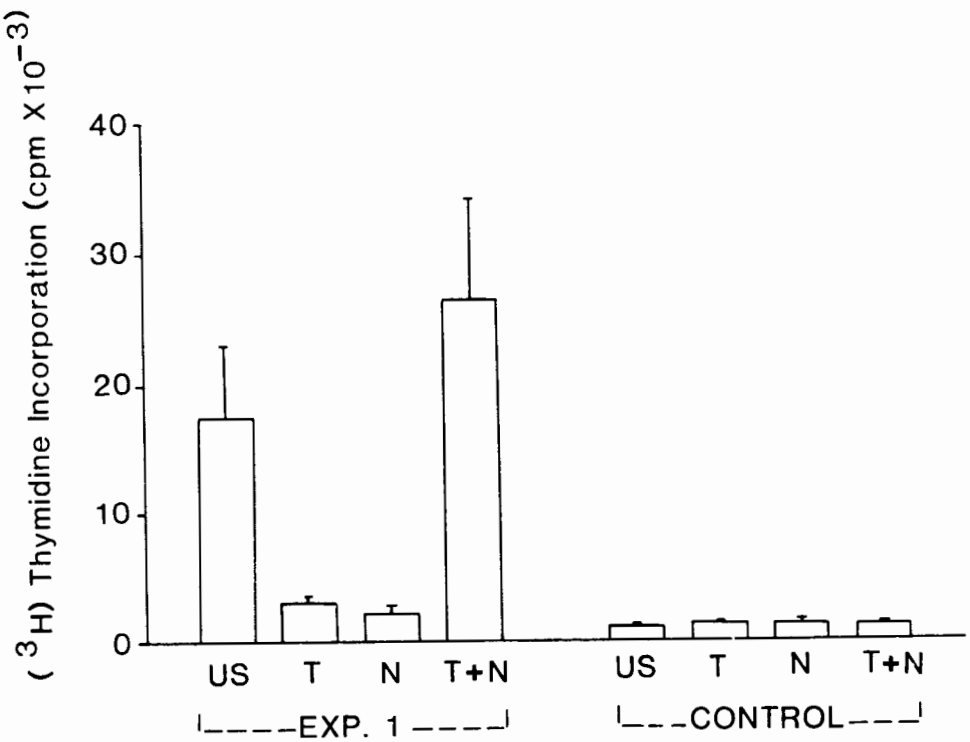
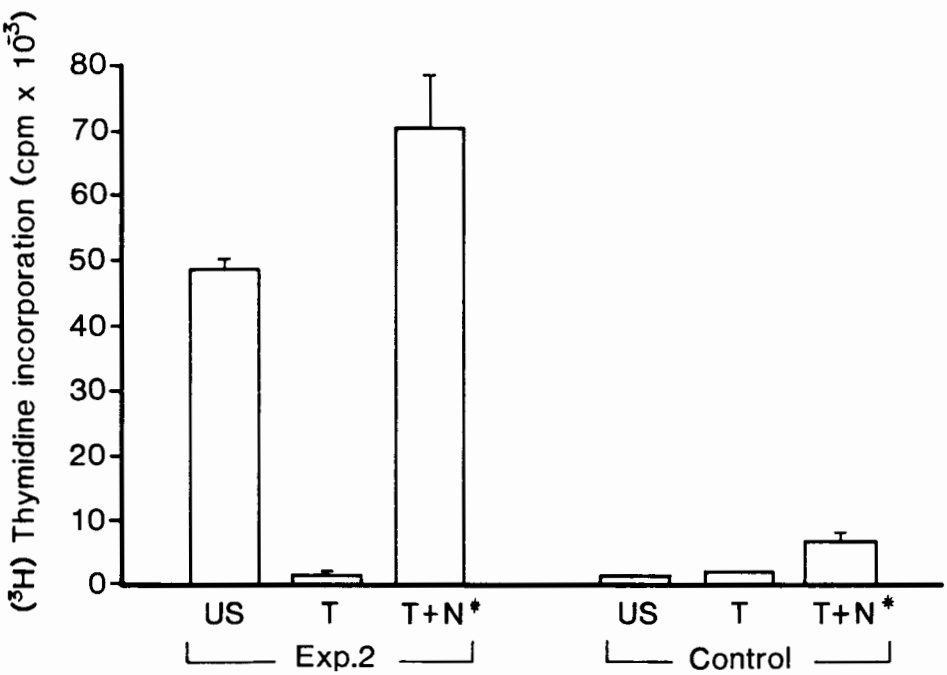


Fig. 2.4.2. Effect of adding back non-T (E^-) cells to purified T (E^+) cells. PBMC were rosetted using SRBC. 10^5/well each of unseparated (US), non-T (N) and purified T cells (T) were cultured in parallel. 5 x 10^4 non-T cells were added back to 5 x 10^4 purified T cells.
* irradiated (1000 rads).



Addition of adherent cell supernatants obtained from patients with high spontaneous proliferation to autologous NAC resulted in marked proliferation (Table 2.7.1). Proliferation was not seen when these supernatants were added to NAC obtained from a normal individual (control 1 in Table 2.7.2). NAC from a TB patient without spontaneous proliferation also did not proliferate in response to the adherent cell supernatant (control 2 in Table 2.7.2). However, the adherent cell supernatant from one patient with high spontaneous proliferation caused the NAC from a second patient with the same phenomenon to proliferate (patient 2 in Table 2.7.1).

2.4.10. Analysis of adherent cell supernatants in the thymocyte co-mitogenesis assay.

Bioassay analysis of the supernatants demonstrated spontaneous thymocyte co-stimulatory activity in adherent cell supernatants obtained from patients with spontaneous proliferation (Table 2.8.1). However, some TB patients without spontaneous proliferation also demonstrated similar levels of spontaneous thymocyte co-stimulatory activity (Table 2.8.2).

2.4.11. Effect of adding adherent cell supernatants to unseparated and purified T cells obtained from a known normal PPD responder

Addition of adherent cell supernatants obtained from patients with high spontaneous proliferation to unseparated control cells obtained from a known PPD responder resulted in proliferation (Fig 2.5). However, addition of the same supernatants to *purified* T cells obtained from the same control individual did not result in proliferation (Fig 2.5). Adherent cell supernatants obtained from normal controls and TB patients without high spontaneous

Table 2.7.1. Effect of adherent cell supernatants obtained from patients with spontaneous lymphocyte proliferation on autologous NAC and NAC obtained from other patients with spontaneous lymphocyte proliferation.

TEST SUPERNATANT		NON ADHERENT CELLS		
		PAT.[+]1	PAT.2	PAT.3
MEDIUM*		9193 ± 1934	1175 ± 343	392 ± 306
PAT.1	EXP. 1	108013 ± 17393	ND[#]	ND
	EXP. 2	111065 ± 19560	ND	ND
PAT.2		23993 ± 6592	23390 ± 5932	ND
PAT.3		ND	ND	56845 ± 3676

Adherent cell supernatants were added to 1 x 10^5 NAC. Proliferation was assessed on day 6 by [^{3}H]thymidine incorporation. Values are mean cpm ± SD. Data represent the means of triplicate determinations.

*MEDIUM, RPMI supplemented with 10% pooled AB human serum.

[+]PAT., patient

[#]ND, not done.

Table 2.7.2. Effect of adherent cell supernatants obtained from patients with spontaneous lymphocyte proliferation on control NAC.

TEST SUPERNATANT	NON ADHERENT CELLS	
	CONTROL 1	CONTROL 2
MEDIUM*	7323 ± 1191	345 ± 25
PAT.+1 EXP. 1	7331 ± 1534	343 ± 25
EXP. 2	4734 ± 1091	ND#
PAT.2	ND	434 ± 116
PAT.3	ND	394 ± 84

Adherent cell supernatants were added to 1 x 10^5 NAC. Proliferation was assessed on day 6 by [^{3}H]thymidine incorporation. Values are mean cpm ± SD. Data represent the means of triplicate determinations.

*MEDIUM, RPMI supplemented with 10% pooled AB human serum.

+PAT., patient

#ND, not done.

Table 2.8.1. Thymocyte co-mitogenic activity of unstimulated adherent cell supernatants obtained from patients with spontaneous lymphocyte proliferation.

TEST SUPERNATANT	MEAN CPM ± SD	STIMULATION INDEX
A] MEDIUM	1070 ± 673	
MEDIUM + PHA	960 ± 436	
PAT. 1 + PHA	3897 ± 300	4.0
PAT. 2 + PHA	8518 ± 1566	8.8
PAT. 3 + PHA	2656 ± 863	2.8
B] MEDIUM	691 ± 138	
MEDIUM + PHA	1555 ± 443	
LPS STIMULATED MONOCYTE SUP. + PHA	18604 ± 2905	12.0
CONTROL MONOCYTE SUP. + PHA	2485 ± 824	1.6

Results are expressed as mean cpm ± SD [^{3}H]thymidine incorporation. Data represent the means of triplicate determinations. Stimulation indeces were calculated as follows: mean cpm in wells stimulated by test supernatant and PHA/mean cpm in wells containing medium and PHA. A stimulation index of greater than 3 was considered a positive result.

PAT.=PATIENT

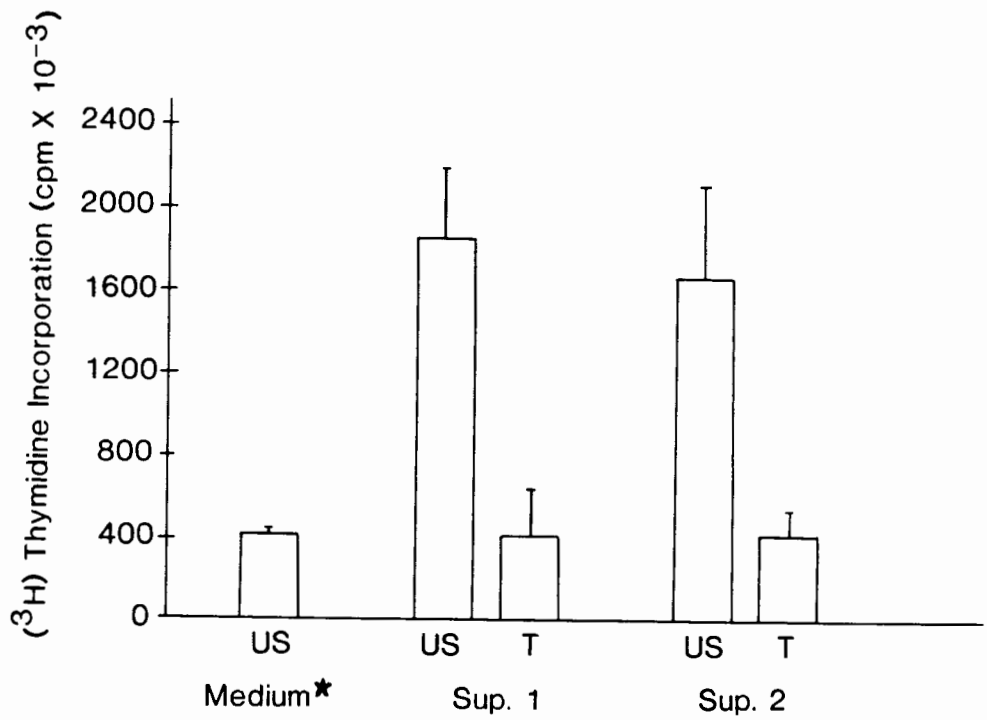
Table 2.8.2. Thymocyte co-mitogenic activity of unstimulated adherent cell supernatants obtained from normals and patients without spontaneous lymphocyte proliferation.

TEST SUPERNATANT	MEAN CPM ± SD	STIMULATION INDEX
MEDIUM	1070 ± 673	
MEDIUM + PHA	960 ± 436	
PAT. 1 + PHA	4634 ± 1224	4.8
PAT. 2 + PHA	4719 ± 118	4.9
PAT. 3 + PHA	2441 ± 570	2.5
PAT. 4 + PHA	761 ± 303	0.8
PAT. 5 + PHA	902 ± 530	0.9
PAT. 6 + PHA	4930 ± 353	5.1
PAT. 7 + PHA	807 ± 241	0.8
PAT. 8 + PHA	1147 ± 448	1.2
NORMAL 1 + PHA	1097 ± 256	1.1
NORMAL 2 + PHA	1280 ± 375	1.3
NORMAL 3 + PHA	1098 ± 198	1.1

Results are expressed as mean cpm ± SD [^{3}H]thymidine incorporation. Data represent the means of triplicate determinations. Stimulation indeces were calculated as follows: mean cpm in wells stimulated by test supernatant and PHA/mean cpm in wells containing medium and PHA. A stimulation index of greater than 3 was considered a positive result.

PAT.=PATIENT

Fig. 2.5. Effect of adding adherent cell supernatants obtained from patients with spontaneous proliferation to unseparated (US) and purified T (T) cells obtained from a known normal PPD responder. Adherent cell supernatants from two patients (SUP.1 and 2) were added to 1 x 10^5 unseparated control PBMC and cultured for 6 days. The same supernatants were added to 1 x 10^5 purified T cells in a parallel culture. The purified T cells were depleted of antigen presenting cells by twice rosetting with SRBC and adhering to plastic for 90 minutes. Proliferation was assessed by [^{3}H]thymidine incorporation into DNA.
* complete medium



proliferation did not stimulate either unseparated or purified T cells (Table 2.9).

2.5. DISCUSSION AND CONCLUSIONS

This is the first description of persisting spontaneous proliferation of peripheral blood lymphocytes in tuberculosis. The proliferation was dependent on the major T cell lymphokine IL-2 as evidenced by the presence of IL-2 receptors on the proliferating cells and the ability to block with anti-IL-2-receptor antibody. The phenomenon was unlikely to be an effect of anti-tuberculous drugs since four of these patients received no therapy prior to inclusion in the study. In one case the proliferation persisted long after therapy was stopped. The data in Table 1 indicates that a serum factor is unlikely since no change in the proliferation was observed when either autologous or pooled serum was used. The concentration of the serum also did not affect the proliferation (Table 2.1). The proliferation was also unlikely to be dependent on accessory cell derived prostaglandins since indomethacin had no effect on the proliferation (Table 2.2).

Several levels of evidence implicate a role for persisting mycobacterial components in the initiation of this phenomenon. Firstly, the kinetics of the proliferation mirrored that of antigen driven proliferation (Table 2.5). Secondly, the proliferation had a strict requirement for APC since : (i) adherent cell depletion abrogated the proliferation (Table 2.6) and (ii) adherent cell supernatants obtained from patients with high spontaneous proliferation caused significant proliferation only upon addition to unseparated control cells (Fig.2.5). The same supernatants did not stimulate highly purified T cells from the same control individual, suggesting a requirement for antigen presentation (Fig.2.5). Thirdly, adherent cell

Table 2.9. Effect of adherent cell supernatants obtained from patients without spontaneous proliferation on unseparated and purified T cells obtained from a known PPD responder control.

TEST SUPERNATANT	MEAN CPM [^{3}H]THYMIDINE INCORPORATION: UNSEPARATED CELLS	PURIFIED T CELLS
MEDIUM	1397 ± 386	819 ± 61
1	1911 ± 283	1013 ± 154
2	1630 ± 239	1420 ± 110
3	2435 ± 682	1750 ± 242

Adherent cell supernatants were added to unseparated and purified T cells obtained from a known PPD responder and proliferation was assessed by [^{3}H]thymidine incorporation on day 6. Data represent the mean ± SD of triplicate determinations.

supernatants obtained from TB patients without spontaneous proliferation were inactive when added to unseparated control cells, despite similar levels of thymocyte co-stimulatory activity to supernatants obtained from patients with spontaneous proliferation (Tables 2.8.1 and 2.8.2). It is well known that the thymocyte co-mitogenesis assay detects several lymphokines including interleukins 1 and 6. The data therefore indicates that the spontaneous proliferation was unlikely to be due to the direct effect of the cytokines alone. The most direct explanation for these data is the presence of antigen in the supernatant. Indeed, a recent *in-vitro* study confirmed the presence of processed antigenic determinants in supernatants of monocytes stimulated with optimal concentrations of mycobacterial antigen (127). Finally, the presence of antigen would also account for the observation that active adherent cell supernatants only caused proliferation when added to NAC obtained from patients with high spontaneous proliferation (Table 2.7.1), and not when added to control NAC (Table 2.7.2). It is likely that the non-rigorous separation techniques employed in this study left residual accessory cells within the NAC population. These accessory cells may be capable of efficiently presenting antigen in spite of their low numbers. It is interesting to note that augmented antigen presentation is a feature of sarcoidosis (109), another granulomatous disorder associated with increased spontaneous proliferation (105,106). An alternative explanation is that the peripheral blood T lymphocytes in these patients have been primed to mycobacterial antigen in vivo, despite the inability to detect the classical activation markers HLA-DR and TAC. Primed T cells, unlike naive cells, can be triggered to proliferate in the absence of antigen presenting cells (128,129). Furthermore, even cloned T lymphocytes do not show a direct correlation between signal requirements for proliferation and HLA-DR or TAC expression (129,130).

Persuasive experimental evidence indicates that even readily degradable protein antigens can indeed persist for months or years in the spleens and draining lymph nodes of immunized animals (100). Persisting antigen at these sites are thought to play a role in the maintenance and regulation of humoral immunity (101). Persisting antigen has also been implicated as a potential aetiological agent in a number of chronic diseases such as rheumatoid arthritis and chronic reactive arthritis following infection with Borrelia or Yersinia (102-104,110).

Spontaneous proliferation is also a feature of rheumatoid arthritis (110). Significant differences exist in the spontaneous proliferation noted in both rheumatoid arthritis and sarcoidosis when compared to the findings in this study. Firstly, in both rheumatoid arthritis and sarcoidosis the proliferation is noted at the site of pathology (synovial fluid (SF) and broncho-alveolar lavage (BAL) fluid respectively) as opposed to peripheral blood in the TB patients discussed herein. Secondly, the proliferation is apparently independent of antigen presenting cells and is present in highly purified T cells in both diseases (105,106,110). Thirdly, fresh lymphocytes obtained from the SF and BAL were found to express increased levels of activation markers such as HLA DR and TAC (109,110). These differences may be the result of *in-vivo* antigen presentation since interaction between T lymphocytes and antigen presenting cells is more likely at the site of the pathology than in the circulating peripheral blood. In the case of the TB patients, it is likely that *in-vitro* culture provides the appropriate environment for interaction between T cells and antigen presenting cells. This would result in the proliferation noted during *in-vitro* culture and account for the absence of activation markers on freshly isolated cells. It is likely that the spontaneous proliferation observed *in-vitro* in TB peripheral blood occurs *in-vivo* at

the site of pathology. In fact, spontaneous proliferation has been noted in unstimulated lymphocytes obtained from some pleural effusions (Table 6.1).

Spontaneous proliferation has also been noted in hybridomas obtained from neonatal thymocytes (90). These hybridomas have been shown to express the gamma-delta T cell receptor and to react to the 65 kDa HSP (90). A considerable percentage of cells bearing this receptor, in the peripheral blood, respond to mycobacterial antigen. The antigen responsible for the spontaneous proliferation noted in the TB PBL may therefore be mycobacterial 65 kDa HSP or the human homologue.

The case represented by Fig.2.2.3 was particularly interesting. This patient had documented pulmonary TB four years previously which was fully treated. At the time of study, only non-specific symptoms of malaise and loss of weight were present. Persistent spontaneous proliferation was demonstrated over a 14 month period in the peripheral blood. Adherent cell supernatants from this patient was able to stimulate proliferation in control cells only in the presence of APC and, in addition, was able to sensitize targets for lysis by PPD-specific CTL (Sup.2 in Fig.2.5 and 2.6). These observations strongly implicate the presence of mycobacterial antigen in this adherent cell supernatant. Although subclinical reactivation is possible, it cannot be excluded that the proliferation in this case may reflect an ongoing immune response dating to the original infection four years previously. The patient represented in figure 2.2.1 was similar in that proliferation persisted for at least four months after all clinical and radiological evidence of disease had disappeared.

Five of the six patients with spontaneous proliferation had extensive disease. Three had disseminated disease, one had

extensive pulmonary disease with resistant organisms and the fifth case had suspected miliary TB. Three of these patients demonstrated poor response to therapy and were unwell for 12 months or longer. The slow clinical resolution observed together with the demonstration that antigen persisted in these patients suggests that these patients have defective immunity. It is likely that phagocytes in these patients have poor microbicidal activity. In addition, it is also possible that these patients have decreased CTL activity. Unfortunately, due to technical considerations and limited access to these patients, these possibilities could not be pursued.

The possibility that antigen was persistently secreted by circulating adherent cells is intriguing since these cells have a short lifespan in the peripheral blood (131). It is possible that a long-lived peripheral pool of adherent cells exist. A similar hypothesis was invoked to explain the long term effects of IFN-Ǒ adminstration on circulating phagocytes in patients with chronic granulomatous disease (132). An alternative possibility is that mycobacterial antigen was being continuously seeded out into the circulation from infected tissue sites. Thus competent cells entering the circulation would be exposed to mycobacterial antigen. It is also possible that the bone marrow in these patients was infected. Mature cells already carrying mycobacterial antigen would then be released into the circulation .

In conclusion, this is the first description of persistent, IL-2 dependent, spontaneous T lymphocyte proliferation in tuberculosis. Previous immune abnormalities, namely necrotizing skin test responsiveness, abnormalities of glycosylation of the F_C portion of IgG, and increased $1,25(OH)_2D_3$ production have been shown to persist in patients with TB. The underlying mechanism for these

inexplicable immune phenomena is not known. The data described here represents a fourth persisting immune abnormality, and provides evidence that it may be due to persistent antigen secretion by adherent cells. Similar observations in rheumatoid arthritis and sarcoidosis suggest that these diseases may also represent ongoing inflammatory and immune responses to persisting antigen. Finally, our observations provide additional support for the concept that mycobacteria persist in the host in some form and are never totally eliminated (134).

CHAPTER 3

CYTOTOXICITY BY PPD-STIMULATED LYMPHOCYTES: A COMPARISON OF THE CONVENTIONAL 4-HOUR ^{51}CHROMIUM RELEASE ASSAY WITH THE EXTENDED 15-HOUR CHROMIUM RELEASE ASSAY

3.1. ABSTRACT

M. tuberculosis is a bacterial pathogen capable of survival and replication within human macrophages. CTL are thought to be important for the eradication of infected macrophages. To test this hypothesis, two assays were analyzed for the purpose of measuring *in-vitro* PPD-specific cytotoxicity. PBL from control PPD-responsive subjects were stimulated *in-vitro* with PPD, and assayed for their cytotoxic capacity in conventional 4-hour ^{51}chromium release assays. Autologous phytohaemagglutinin stimulated blasts maintained in IL-2 were used as targets. No significant PPD-specific lysis could be demonstrated using this system. Significant lysis could however be demonstrated by using a 15-hour chromium release assay which utilized autologous adherent cells as targets.

3.2. INTRODUCTION

The hypothesis that CTL are important for protection against mycobacteria is an attractive one. It is known that mycobacteria may persist within parasitized macrophages (12). IFN-Ǒ activated macrophages have not been convincingly shown to inhibit growth of *M. tuberculosis* in humans (30-37). Lysis of parasitized macrophages by specific CTL would destroy the organisms environment and expose them to more hostile macrophages as well as alternative immune mechanisms (12). A failure in such a mechanism could then conceivably result in persistence of the infection and ongoing immune

activation as described in chapter 2. As a result of technical difficulties (particularly ill patients who could not be repeatedly venesected for the provision of target cells) this hypothesis could not be pursued further in the patients with spontaneous proliferation. As an alternative, the general hypothesis that cell mediated cytotoxicity was important for protection against mycobacteria was investigated in control subjects and patients with tuberculous pleural effusions.

To date, significant PPD-specific cytotoxicity has been demonstrated in only one human study when the conventional 4-hour chromium release assay was used (43). Most other studies have used a 15-hour chromium release assay which employs adherent cell targets obtained from the peripheral blood (46-48). Here, the two methods are compared to standardise an assay system for the measurement of PPD-specific cytotoxicity.

3.3. MATERIALS AND METHODS

3.3.1. Cell separation and preparation of effectors

PBMC were obtained from control PPD responders by Ficoll-Hypaque density gradient centrifugation as described in chapter 2.3.3. The PBMC were stimulated with 3µg/ml PPD (Connaught Laboratories, Willowdale, Ontario, Canada) for 6 days.

3.3.2. 4-hour chromium release assay

Targets were autologous Phytohaemagglutinin (PHA, H A 16, Welcome Diagnostics, Temple Hill, Dartford, England) stimulated blasts maintained in IL-2. Targets ($2\text{-}4 \times 10^6$) were labelled with 250 µCi ^{51}sodium chromate (specific

activity 3.7-13Gq/mg, Amersham International, Amersham, U.K.) at 37°C for 1 hour in 0.1 ml of medium. The targets were washed 3 times in PBS and labelled with varying concentrations of PPD (ranging from 10µg to 50µg/ml) for 3 hours on the day of the assay or overnight prior to the assay. Some target cells were labelled with PPD both overnight prior to the assay and for 1 hour on the day of assay. Control target cells were not labelled with antigen or were labelled with streptokinase-streptodornase (SK-SD, 1:100 dilution, Lederle Lab., Wayne, N.J., U.S.A.). The assay was performed in triplicate in U-bottomed microtitre plates (Flow Laboratories) in complete medium. Labelled target cells were plated at 3 x 10^3 per well and effectors were added to target cells at varying E/T ratios in a final volume of 0.2 ml per well. Some wells contained target cells and complete medium without effectors for the determination of spontaneous release. Triton-X (5%, BDH 30632, BDH LTD, Poole, England) was added to some wells for the determination of maximum chromium release. Plates were spun at 150G for 5 minutes and incubated at 37°C for 4 hours after which 130 µl of the supernatant from each well was removed into disposable counting tubes (Greiner, Nurtingen, West Germany) and counted in a gamma counter (Packard, auto-gamma scintillation spectrometer). The percentage specific release was calculated by: 100 x [(mean cpm experimental) - (mean cpm spontaneous release)]/[(mean cpm maximum release) - (mean cpm spontaneous release)].

3.3.3. 15-hour chromium release assay

The cytotoxic capacity of effector cells which had been stimulated for 4 or 6 days with PPD in 24 well tissue culture plates (Nunc, Kamstrup, Denmark) was assayed as previously described (47). Target cells were day 4 or day 6 autologous adherent cells of PBMC that had been plated in 96-well U-bottomed tissue culture plates (Nunc, Kamstrup,

Denmark). Approximately 10% adhered. This number was used for calculating E/T ratios. One day prior to assay the targets were pulsed with antigen (PPD 10µg/ml or SK-SD 1:100 dilution) and labeled with 6 µCi of ^{51}sodium chromate (specific activity 3.7-13Gq/mg, Amersham International, Amersham, UK) during 18 hours culture at 37 ^{o}C and then washed three times with preheated (37 ^{o}C) medium. Effector cells (100 µl/well) were then added in triplicate to the target cells at E/T ratios of 10, 3 and 0.3. Wells containing medium only were used for the determination of spontaneous release. The assays were then incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. 15 hours later the total supernatant content of each well was transferred to a disposable counting tube (Greiner, Nurtingen, West Germany) and 100µl of triton-X was added to the remaining adherent cells for the determination of maximum release. After 3 hours the total volume of triton-X was transferred to similar tubes and the samples were counted in a gamma counter (Packard, auto-gamma scintillation spectrometer). The percentage-specific killing for the mean of triplicate wells was calculated as follows: percent specific lysis = [mean test cpm/(mean test cpm + mean cpm after triton-X treatment of the same triplicate wells)] x 100% - percent spontaneous release. The percentage spontaneous release was calculated as follows: mean cpm in spontaneous release wells/(mean cpm in spontaneous release wells + mean cpm after triton-X treatment of the same triplicate wells) x 100%. Cytotoxicty was considered significant if greater than 5% (133).

3.4. RESULTS

3.4.1. Technical data

In the 15-hour assay, the mean percentage spontaneous release (± SD) of chromium by all targets was 18 ± 5 (n =

92). The mean percentage spontaneous release (± SD) from targets treated with PPD and SK-SD was 19 ± 5 (n = 22) and 18 ± 5 (n = 22) respectively. The mean percentage spontaneous release (± SD) by untreated targets was 20 ± 6 (n = 22) . Similar results were obtained in the 4-hour assay.

3.4.2. Failure to demonstrate significant cytotoxicity with the conventional 4-hour chromium release assay

No significant lysis was obtained in the majority of experiments when the conventional 4-hour chromium release assay was used (Tables 3.1.1, 3.1.2 and 3.2). In the minority of experiments, low levels of cytotoxicity were observed at 4 hours (Tables 3.1.1, 3.1.2, 3.2 and 3.3). No significant increase in lysis was observed even when PBL targets were pulsed with antigen for extended periods (Table 3.2). Similarly no increase in lysis was observed when higher E/T ratios were used, or when higher doses of antigen were used to pulse targets (Tables 3.2 and 3.4). Significant antigen-specific lysis was observed when the 15-hour chromium release assay was used (Tables 3.1 and 3.3).

3.4.3. Comparison of cytotoxicity at 4, 7 and 15 hours in the assay employing adherent cell targets

Only low levels of cytotoxicity were observed at 4 and 7 hours. Significant cytotoxicity was observed at 15 hours (Table 3.3).

3.4.4. Cytotoxicity against targets pulsed with varying doses of antigen in the 15-hour assay

No significant difference in cytotoxicity was observed over the dose range employed for labelling of targets in the 15-hour assay (Table 3.5).

Table 3.1.1. Mean PPD-specific cytotoxicity of ten control subjects as measured in the 15-hour chromium release assay compared to mean PPD-specific cytotoxicity in 16 experiments utilizing the 4-hour chromium release assay.

		% SPECIFIC LYSIS OF TARGETS PULSED WITH:		
Assay type	E/T ratio	PPD	SK-SD[+]	NO Ag[*]
15-hour	10	39 ± 5	8 ± 2	6 ± 3
	3	31 ± 5	4 ± 1	4 ± 2
	0.3	13 ± 4	1 ± 1	3 ± 2
4-hour	10	3 ± 1		
	3	2 ± 1		
	1	1 ± 1		

Results are expressed as mean percentage specific lysis ± SE of 10 and 16 experiments respectively. All the subjects used were known PPD responders.

[+]SK-SD, streptokinase-streptodornase

[*]Ag, antigen

Table 3.1.2. Cytotoxicity by PPD-stimulated effectors obtained from a *single* subject in a conventional 4-hour chromium release assay compared to a 15-hour chromium release assay.

		% SPECIFIC LYSIS OF TARGETS PULSED WITH:		
Assay type	E/T ratio	PPD	SK-SD+	NO Ag*
4-hour	10	7	0	3
	3	0	0	0
	1	0	0	0
	0.3	0	0	0
15-hour	10	36	2	0
	3	27	0	0
	0.3	8	0	0

Results are expressed as the mean percentage specific lysis of triplicate cultures as described for each assay type in materials and methods. SD were mostly less than 10%.

Effector and target cells for both assays were obtained at the same time from a single control subject. Effectors were stimulated for 6 days with 3 µg/ml PPD and targets were labelled as described in materials and methods.

+SK-SD, streptokinase-streptodornase
*Ag, antigen

Table 3.2. Effect of extended antigen pulsing of target cells, on cytotoxicity, in conventional 4-hour chromium release assays.

Exp. No.	E/T ratio	% SPECIFIC LYSIS OF TARGETS PULSED WITH PPD FOR: 3 hour	O/N*	O/N + 1 hour#
1	10	3	ND+	4
	3	0	ND	3
	0.3	0	ND	2
2	10	ND	2	7
	3	ND	1	8
	0.3	ND	0	4
3	20	ND	ND	3
	10	ND	ND	4
	3	ND	ND	6
	1	ND	ND	1
4	20	ND	ND	4
	10	ND	ND	2
	3	ND	ND	1
	1	ND	ND	0

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%.

*O/N, overnight

#O/N + 1 hour, targets incubated with PPD overnight and treated again with PPD the following morning for one hour

+ND, not done

Table 3.3. PPD-specific cytotoxicity at 4, 7, and 15 hours in the chromium release assay employing adherent cell targets.

	% SPECIFIC LYSIS OF TARGETS PULSED WITH:	
	PPD	NO Ag*
EXP.1:		
4 HOURS	4	2
7 HOURS	3	2
15 HOURS	20	3
EXP.2:		
4 HOURS	11	7
15 HOURS	35	3

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%.
All experiments were performed at an effector to target ratio of 10:1.
*Ag, antigen

Table 3.4. PPD-specific cytotoxicity in the conventional 4-hour chromium release assay against target cells pulsed with varying concentrations of antigen.

	PERCENT SPECIFIC LYSIS OF TARGETS PULSED WITH:	
DOSE (μg/ml)	PPD	No antigen
100	2	1
50	0	0
25	0	0
12	3	1
6	1	3
3	0	3

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%. All experiments were performed at an effector to target ratio of 10:1.

Table 3.5. Effect of target cells pulsed with varying concentrations of PPD antigen, on cytotoxicity, in the 15-hour chromium release assay.

DOSE (μg/ml)	PERCENT SPECIFIC LYSIS OF TARGETS PULSED WITH: EVANS* PPD	CONNAUGHT PPD
100	10	ND
50	10	10
25	10	10
12	7	9
6	10	9
3	ND	10

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%.
Effectors were PBL (obtained from a control subject) stimulated with Connaught PPD for 6 days.
All experiments were performed at an effector to target ratio of 3:1.
* Evans PPD (Evans corporation, England, United Kingdom)

3.5. DISCUSSION AND CONCLUSIONS

The data indicates that only low levels of cytotoxicity were generated at 4 hours irrespective which assay system was employed. The failure to demonstrate cytotoxicity in the 4-hour assay was clearly confirmed by the experiment illustrated in Table 3.1.2. In this experiment, a single culture of effector cells was appropriately divided between the two assay types and identical E/T ratios were used. The targets were also treated with the same amount of antigen. At an E/T ratio of ten, only 7% lysis was noted against autologous PBL targets treated with PPD in the 4-hour assay as compared to 36% lysis directed at autologous adherent cell targets treated with PPD in the 15-hour assay.

The low levels of lysis observed in the 4-hour assays were unlikely to be due to too brief an exposure of target cells to antigen since even extended periods of exposure to antigen at high concentrations made no difference to the levels of cytotoxicity (Tables 3.2 and 3.4). Furthermore, minute amounts of antigen were adequate to sensitize targets for antigen-specific lysis in the 15-hour assay (Table 3.5). The cytotoxicity was mostly directed at targets treated with PPD and was highest at high E/T ratios. Low levels of cytotoxicity was directed at untreated targets or targets treated with SK-SD. The non-specific kill was greater at the highest E/T ratios and was almost certainly NK or LAK mediated. This was confirmed later (Chapter 4).

Both assay systems were found to be technically reliable and reproducible. Targets were successfully chromated in both assay systems. Poor chromation and poor viability (as assessed by trypan blue exclusion) precluded the use of resting PBL as targets as was done in a previous study (43). Spontaneous release of chromium was considered acceptable if less than 30% of maximum release after addition of Triton-X.

This was much more stringent than was recommended (133). From the results it is clear that pre-incubation with antigen did not affect percentage spontaneous release. Preliminary experiments with the 15-hour assay demonstrated no significant differences in percentage spontaneous release when day 4, day 6 or fresh PBL adherent cells were employed as targets. Cytotoxicity was also not significantly different. Thus day 4 or day 6 adherent cells were used in most experiments as this required a single venesection of the subject under study.

The cytotoxicity observed at 15 hours, in the assay employing autologous adherent targets, is mediated by $CD4^+$ CTL (section 4.4.5). The mechanism of cytotoxicity utilized by $CD4^+$ CTL may possibly account for the delay observed before significant cytotoxicity was detected.

Cytotoxicity observed at 4 hours in the conventional chromium release assay has in most cases been shown to be mediated by $CD8^+$ CTL (52,55). $CD8^+$ CTL recognize antigen in association with MHC class I products (52,55). Exogenously added antigen usually enters the class II pathway of antigen processing (52,55). This may therefore account for the failure to demonstrate cytotoxicity at 4 hours. This possibility is addressed in chapter 5.

CHAPTER 4

CELL MEDIATED CYTOTOXICITY IN TUBERCULOSIS: A COMPARISON OF CYTOTOXICITY BY PLEURAL EFFUSION, AUTOLOGOUS PERIPHERAL BLOOD AND CONTROL PERIPHERAL BLOOD LYMPHOCYTES

4.1. ABSTRACT

Numerous theoretical considerations suggest that CTL are important in the immune response to *M. tuberculosis*. To pursue this hypothesis, the level and kinetics of generation of antigen-specific cytotoxicity of *in-vitro* PPD-stimulated PEL was determined using 15-hour ^{51}chromium release assays. Cytotoxicity in PEL obtained from patients with tuberculous pleuritis was compared with that in cells obtained from autologous peripheral blood, control peripheral blood and non-tuberculous effusions. In 6 tuberculous pleural effusions, both proliferation and cytotoxicity were augmented and accelerated in comparison to autologous or control peripheral blood. By contrast, low levels of cytotoxicity with conventional kinetics of induction were observed in non-tuberculous effusions. Cell subset fractionation experiments indicated that the cytotoxicity was mediated by $CD4^+$ cells. The accelerated kinetics of induction of PPD-specific CTL demonstrated here suggests reactivation of *in-vivo* generated CTL. These findings provide evidence that CTL are induced at the site of pathology *in-vivo*, and suggest that these cells have a role in *in-vivo* protection against infection with tuberculosis.

4.2. INTRODUCTION

A reliable and reproducible assay system for determining PPD-specific cytotoxicity was established in Chapter 3. In this section the objective was to apply this assay to assess

cell mediated cytotoxicity in patients with tuberculous pleuritis.

Pleural effusions provide a unique opportunity to study immune function in cells obtained from the site of the pathology. The disease is common and the lymphocytes in the pleural fluid are usually numerous and easily accessible. Furthermore, the viability of these cells as determined by trypan blue exclusion is almost 100%. This contrasted with lymphocytes obtained from BAL fluid where only small numbers of lymphocytes were obtained and viability was low.

Cellular immunity is thought to be effective in tuberculous pleural effusions because many are known to resolve spontaneously (135). This constituted another indication for analyzing T cell function in pleural effusions as this was the site most likely to yield evidence of *in-vivo* cell mediated cytotoxicity. Circumstantial evidence implicating an *in-vivo* role for cell mediated cytotoxicity has been described in Leprosy (46). Such evidence is not available in human tuberculosis.

Proliferation and cytotoxicity was measured on days one, four and six since a significant earlier response would imply a secondary immune response and therefore *in-vivo* priming with mycobacterial antigen. The level of cytotoxicity by pleural effusion lymphocytes was also compared to that in autologous peripheral blood and control peripheral blood as it was argued that cytotoxicity would be augmented, in active disease at the site of the pathology, if it was involved in protective immune responses.

The phenotypic nature of the cell responsible for antigen-specific lysis in the 15-hour assay was established by utilizing cell separation techniques. These cells were shown

to be MHC class II restricted. In addition, PEL NK activity was analyzed in 4-hour chromium release assays to characterize the nature of the antigen non-specific lysis noted in the 15-hour assays.

4.3. MATERIALS AND METHODS

4.3.1. Patient population

Pleural fluid and peripheral blood samples were obtained at the time of closed pleural biopsy in 7 patients with newly diagnosed tuberculous pleural effusions, seen at Groote Schuur Hospital between July and December 1989. Pleural biopsy revealed granulomatous pleuritis in 5 cases (with the presence of *M.tuberculosis* in 1). Tuberculosis was diagnosed in one patient with constrictive pericarditis and pleural effusion on the basis of the clinical picture and response to therapy. In the remaining case *M.tuberculosis* was cultured from the sputum. Pleural fluid was also obtained from 5 patients with non-tuberculous aetiologies (malignancy, 3 cases; parapneumonic effusion, 1 case; non-specific inflammation, 1 case). Peripheral blood samples were also obtained from 10 PPD-responsive control subjects for the analysis of cytotoxicity.

4.3.2. Proliferation assay

Peripheral blood lymphocytes (PBL) and pleural effusion lymphocytes (PEL) were separated on a Ficoll-Hypaque gradient as described in chapter 2, the only modification being that pleural effusions were overlaid directly onto Ficoll-Hypaque without diluting first. PBL or PEL (1×10^5) were resuspended in complete medium and added to wells of 96 well round bottomed sterile tissue culture plates (Flow Laboratories). Cells were stimulated with 3 µg/ml PPD (Connaught Laboratories, Willowdale, Ontario, Canada) except

for some wells which contained responder cells alone for the determination of background proliferation. Cultures were incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 and pulsed with Tritiated thymidine (2 µCi/well, specific activity 185 Mega BQ, Amersham 120, Amersham, U.K.) on day 1, 3 or 6 for the final 16-18 hours of incubation. The cells were harvested using an automated harvester (Titertek 630), and radioactivity was measured using a liquid scintillation counter (Packard, Tricarb 4640). All assays were performed in triplicate. Results were expressed as lymphocyte transformation indexes (LTI). The LTI was calculated as follows: LTI = mean counts per minute in antigen stimulated wells/mean counts per minute in unstimulated wells.

4.3.3. Cytotoxicity assays

The percentage specific chromium release was determined after 4 or 15 hours of incubation. Effector cells (1 x 10^6/well) were stimulated with 3 µg/ml PPD and cultured for 1, 4 or 6 days and utilized in the relevant assays.

4.3.3.1. The 4-hour assay.

Natural killer (NK) activity was evaluated in a standard 4-hour chromium release assay as described in 3.3.2., with the exception that: (1) the E/T cell ratio was 30:1, 10:1, 3:1, and 1:1.; and (2) the K562 cell line served as target cells.

4.3.3.2. The 15-hour assay.

The 15-hour chromium release assay was performed as described in section 3.3.3., with the exception that the assay was also performed using targets and effectors obtained on day 1 of culture. For the results shown in Table 4.5, cytotoxicity was expressed in the number of lytic units

(LU) for each effector cell population, calculated from the linear portion of the dose response curve: 1 LU was defined as the number of effector cells required to cause 25% lysis from 1 x 10^4 adherent monocytes (15-hour assay) or 3 x 10^3 K562 target cells (4-hour assay) (136).

4.3.4. Isolation of T cell subsets

Enriched lymphocyte subsets were obtained by a panning technique as previously described (137). Unfractionated day 5 PPD-stimulated lymphocytes (1 x 10^7) were incubated with Leu 16 (Becton Dickinson, Mountain View, U.S.A.) for 45 minutes at 4°C. The cells were subsequently washed twice in 3%HS-PBS and incubated for 1 hour in petri dishes that were previously coated with affinity purified goat anti-mouse IgG (Tago, Burlingame, California, U.S.A.) at 100 µg/10 ml/petri dish in Tris buffer, pH 9.5. B cells and monocytes (by plastic adherence) were thus removed during this stage. After this preliminary incubation, the cells were washed and incubated with T8 ascites (ATCC). After 45 minutes, the cells were washed twice in 3% HS-PBS and incubated in a 3 ml volume in goat anti-mouse IgG coated petri dishes as above. After 2 hours NAC ($CD4^+$ enriched) were collected. Following 4 gentle washes to remove remaining NAC, the adherent cells ($CD8^+$ enriched) were removed by vigorous pippeting. The cells were incubated overnight at 37°C in complete medium and utilized in cytotoxicity assays the following day.

4.3.5. Immunostaining

T4 or T8 ascites (50 µl, ATCC) was added to 10^6 mononuclear cells suspended in 100 µl of fluorescence mixture (3% FCS-1% Na-azide-PBS). Primary antibody was ommitted from cells serving as a background control for fluorescence. After incubation at 4 °C for 40 minutes, the cells were washed 3 times with fluorescence mixture. A FITC-conjugated goat

anti-mouse IgG was added as a secondary antibody. Cells were washed after 30 minutes and percentage positive cells analyzed by indirect fluorescence microscopy when at least 200 cells were counted.

4.3.6. Statistical analysis

Confidence intervals were calculated as described (126). Analysis of variance by comparison of means was used to calculate P values.

4.4. RESULTS

4.4.1. Comparison of cytotoxicity on day 4 and day 6 by PEL and autologous PBL

In PBL, generally low levels of cytotoxicity were detectable on day 4, and a wide range of cytotoxicity was observed on day 6, (Table 4.1). These kinetics were paralleled by proliferation data which were available for 4 of 6 patients (Table 4.1).

By contrast, the striking finding with PEL was accelerated kinetics with respect to both cytotoxicity and proliferation (Table 4.2). All 6 patients had high levels of cytotoxicity directed at PPD labelled targets, already detectable by day 4 of culture. Cytotoxicity was markedly reduced by day 6 in 2 of the 6 patients and remained on a plateau in the remaining 4 patients. Similarly, proliferative responses to PPD peaked on day 4, although day 6 LTI were markedly elevated compared to PBL.

4.4.2. Level of cytotoxicity by PEL compared to PBL

The mean peak PPD-specific cytotoxicity by PEL of 6 patients

Table 4.1. Cytotoxicity of PPD-stimulated PBL obtained from patients with tuberculous pleuritis.

Patient	Day	LTI*	E/T ratio	PERCENT SPECIFIC LYSIS OF MONOCYTES PULSED WITH: PPD	SK-SD+	NO Ag#
1	4	2	10	9	0	0
			3	0	0	0
			0.3	0	0	0
	6	3	10	39	17	13
			3	12	6	0
			0.3	1	0	2
2	4	5	10	5	6	0
			3	0	0	0
			0.3	0	0	0
	6	16	10	16	7	9
			3	8	4	6
			0.3	0	0	0
3	4	6	ND**			
	6	22	10	7	0	0
			3	3	0	0
			0.3	0	0	0
4	4	14	10	36	18	21
			3	17	0	6
			0.3	10	0	1
	6	66	10	50	31	18
			3	41	15	7
			0.3	15	4	1
5	4	ND	10	20	2	6
			3	9	0	0
			0.3	1	0	0
	6	ND	10	48	0	6
			3	27	0	4
			0.3	7	0	0
6	4	ND	10	16	0	0
			3	6	0	0
			0.3	0	0	0
	6	ND	10	25	5	6
			3	16	3	3
			0.3	9	5	5

FOOTNOTES TO TABLE 4.1.

Results are expressed as the mean percentage specific lysis of triplicate cultures calculated as described in Materials and Methods. SD were mostly less than 10%.

*LTI, lymphocyte transformation index

+SK-SD, streptokinase-streptodornase

#Ag, antigen

**ND, not done

Table 4.2. PPD-stimulated cytotoxicity by tuberculous PEL.

Patient	Day	LTI*	E/T ratio	PERCENT SPECIFIC LYSIS OF MONOCYTES PULSED WITH: PPD	SK-SD+	NO Ag#
1	4	87	10	56	35	34
			3	51	21	25
			0.3	38	14	15
	6	32	10	70	6	7
			3	65	2	0
			0.3	33	0	0
2	4	83	10	66	39	42
			3	45	19	26
			0.3	17	3	2
	6	51	10	62	28	24
			3	42	13	9
			0.3	8	0	0
3	4	52	10	46	4	2
			3	24	0	1
			0.3	5	0	0
	6	27	10	22	1	1
			3	11	0	1
			0.3	1	0	0
4	4	94	10	50	22	22
			3	38	2	5
			0.3	15	0	0
	6	73	10	60	20	13
			3	49	8	1
			0.3	25	1	2
5	4	ND**	10	56	11	8
			3	32	0	0
			0.3	1	0	0
	6	ND	10	11	0	0
			3	0	0	0
			0.3	0	0	0
6	4	ND	10	62	13	19
			3	46	3	0
			0.3	27	0	0
	6	ND	10	60	24	28
			3	35	7	8
			0.3	10	1	1

FOOTNOTES TO TABLE 4.2.

Results are expressed as the mean percentage specific lysis of triplicate cultures calculated as described in Materials and Methods. SD were mostly less than 10%.

*LTI, lymphocyte transformation index

+SK-SD, streptokinase-streptodornase

#Ag, antigen

**ND, not done

with TB was significantly higher than that observed by autologous PBL (Fig. 4.1). At an E/T of 10:1, the difference between the mean peak cytotoxicity in PEL and autologous PBL was 26, with a 95% confidence interval from 8.6 to 43.4; The *t* test statistic was 2.2, with 10 degrees of freedom and an associated P value of $P < 0.01$. At an E/T of 3:1, Mean difference 26, 95% CI 7.1 to 44.9; $t = 2.2$, df = 10, $P < 0.01$. At an E/T of 0.3:1, Mean difference 14, 95% CI 1.1 to 26.9; $t = 2.2$, df = 10, $P > 0.05$. Thus statistical significance was not achieved only at the lowest E/T ratio. The mean peak PPD-specific cytotoxicity in PEL of six patients with TB was also significantly higher than that measured in ten control PPD responders (Fig. 4.2). At an E/T of 10:1, Mean difference 20, 95% CI 11.6 to 28.4; $t = 2.2$, df = 14, $P < 0.01$. At an E/T of 3:1, Mean difference 16, 95% CI 5.7 to 26.3; $t = 2.2$, df = 14, $P < 0.01$. At an E/T of 0.3:1, Mean difference 8, 95% CI -2.9 to 18.9; $t = 2.2$, df = 14, $P > 0.05$. Thus, statistical significance was again not achieved only at the lowest E/T ratio.

4.4.3. Cytotoxicity in non-tuberculous effusions

In effectors obtained from non-tuberculous effusions, generally low levels of antigen-specific cytotoxicity were observed on day 4 (Table 4.3). In patient 1, significant cytotoxicty was observed on day 4 against all three targets. However, cytotoxicity of PPD-labelled targets was not enhanced in spite of a high LTI of 72, in marked contrast to what was seen in TB effusions. In two patients significant PPD-specific cytotoxicity was observed on day 6.

4.4.4. Results of panning

Fluorescence microscopy confirmed negatively selected cells to be 81% and 78% CD4 positive in two experiments. Negatively selected cells were CD8 negative in both

Fig. 4.1. Mean peak PPD-specific cytotoxicity by PEL of 6 patients with TB compared to autologous PBL. Cytotoxicity is expressed as mean ± standard error and is markedly elevated in the PEL.

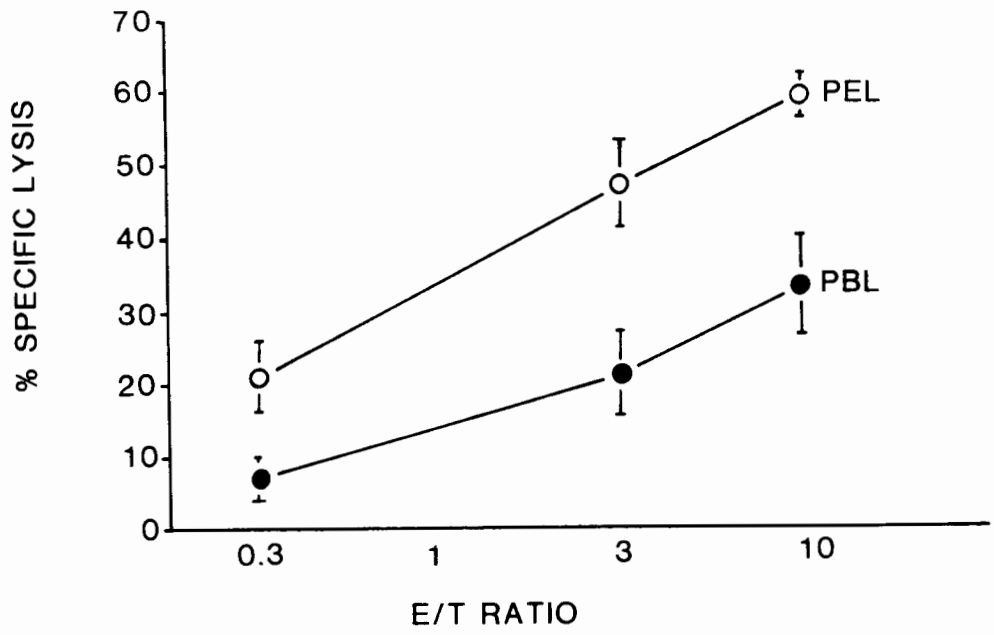


Fig. 4.2. Mean peak PPD-specific cytotoxicity by PEL of 6 patients with TB compared to PBL obtained from 10 control PPD responders. Cytotoxicity is expressed as mean ± standard error and is significantly greater in the PEL.

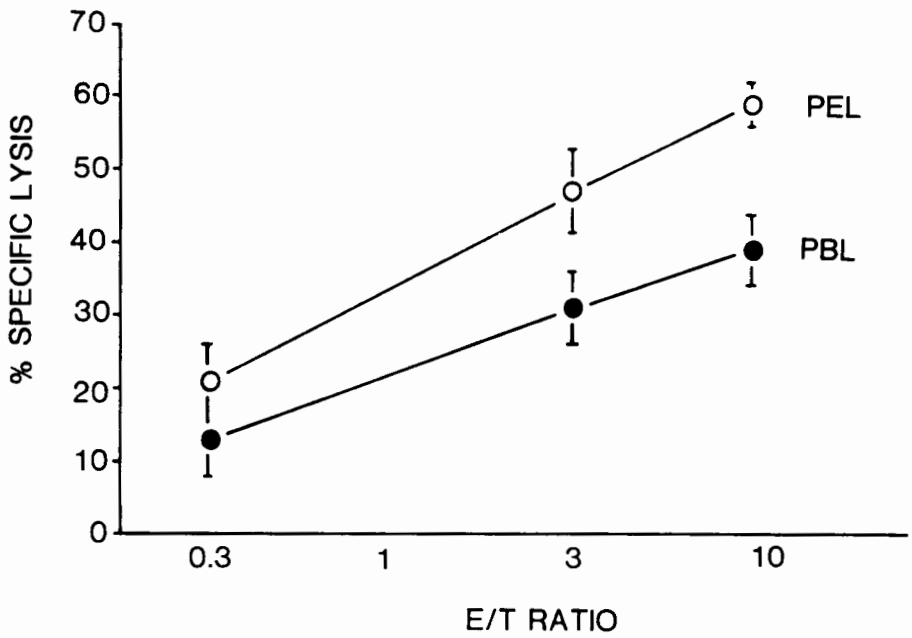


Table 4.3. PPD-stimulated cytotoxicity by non-tuberculous PEL.

Patient	Day	LTI*	E/T ratio	PERCENT SPECIFIC LYSIS OF MONOCYTES PULSED WITH: PPD	SK-SD+	NO ANTIGEN
1	4	72	10	28	34	34
			3	22	21	13
			0.3	7	15	3
	6	77	10	54	13	6
			3	40	5	1
			0.3	14	4	1
2	4	11	ND#			
	6	39	10	29	15	8
			3	25	0	0
			0.3	12	0	2
3	4	5	10	4	ND	ND
	6	7	10	8	8	2
			3	0	5	2
			0.3	0	0	0
4	4	ND	10	5	ND	0
			3	4	ND	0
			0.3	1	ND	0
	6	ND	10	4	0	0
			3	2	0	0
			0.3	1	0	0
5	4	2	10	0	0	0
			3	0	0	0
			0.3	0	0	0
	6	1	10	0	0	0
			3	0	0	0
			0.3	0	0	0

FOOTNOTE TO TABLE 4.3.

Results are expressed as the mean percentage specific lysis of triplicate cultures calculated as described in Materials and Methods. SD were mostly less than 10%.
*LTI, lymphocyte transformation index
+SK-SD, streptokinase-streptodornase
#ND, not done

experiments. Positively selected cells were 56% and 48% CD8 positive respectively. Positively selected cells were CD4 negative in both experiments.

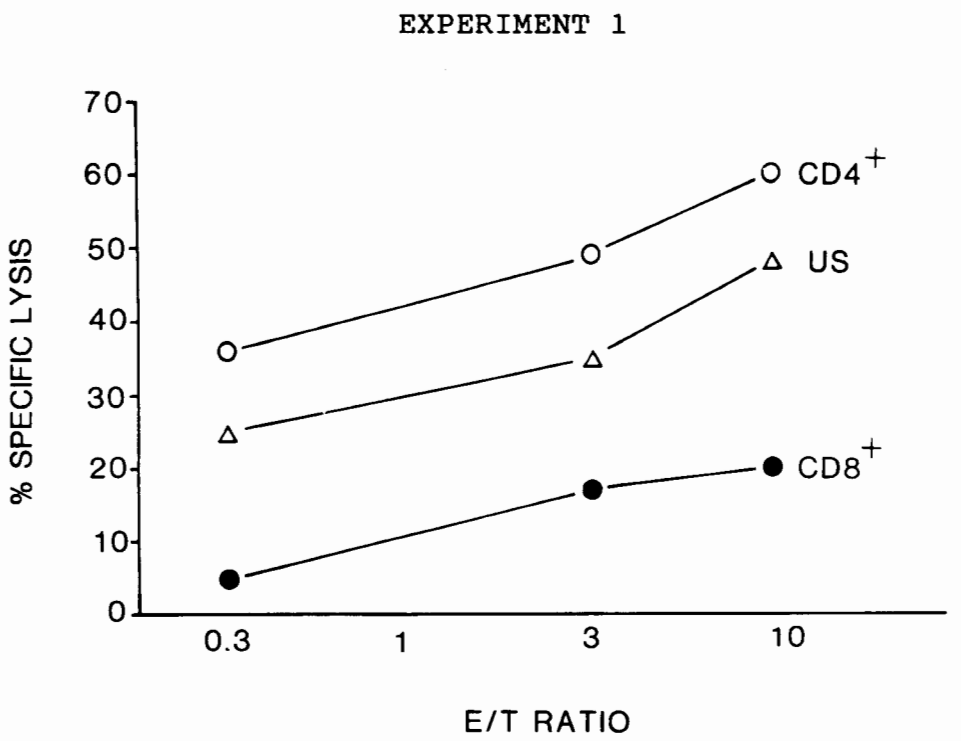
4.4.5. Cytotoxicity by PEL is mediated by $CD4^+$ cells

The cytotoxicity observed in the $CD4^+$ enriched population, negatively selected by panning, was enhanced in comparison to both the unseparated control cells, as well as the positively selected $CD8^+$ subset (Fig. 4.3 and Table 4.4).

4.4.6. Kinetics and specificity of PPD-induced cytotoxicity

In the experiments shown in Tables 4.1-4.3, significant cytotoxicity with little or no antigen non-specific kill was frequently observed at the lowest E/T ratio tested (0.3:1). At higher E/T ratios, lysis of SK-SD-pulsed and unpulsed target cells was observed, and this probably represented natural killer (NK) or lymphokine activated killer (LAK) activity (46). Therefore, the kinetics and specificity of cytotoxic T cell generation, following PPD stimulation, was further evaluated by the simultaneous measurement of NK-activity (4-hour chromium release assay using K562 target cells) and $CD4^+$-mediated cytotoxicity (15-hour chromium release assay) on days 1, 4, and 6 of culture. Representative results from a patient with proven tuberculous pleuritis are shown in Table 4.5. In order to concisely display a large amount of relevant data, results were expressed in lytic units (as defined in Materials and Methods). This provided an overall quantitative expression of the data for the entire dose-response curve for each effector population, and was thus the best quantitative comparison of cytotoxicity. Comparison of % cytotoxicity values at one E/T cell ratio could have been misleading. The following conclusions can be drawn from the data : (i) low

Fig. 4.3. Comparison of cytotoxicity in CD4 and CD8 enriched subsets of PEL obtained from a patient with TB. PPD-specific cytotoxicity is mediated by the CD4 subset. (US = unseparated PEL).



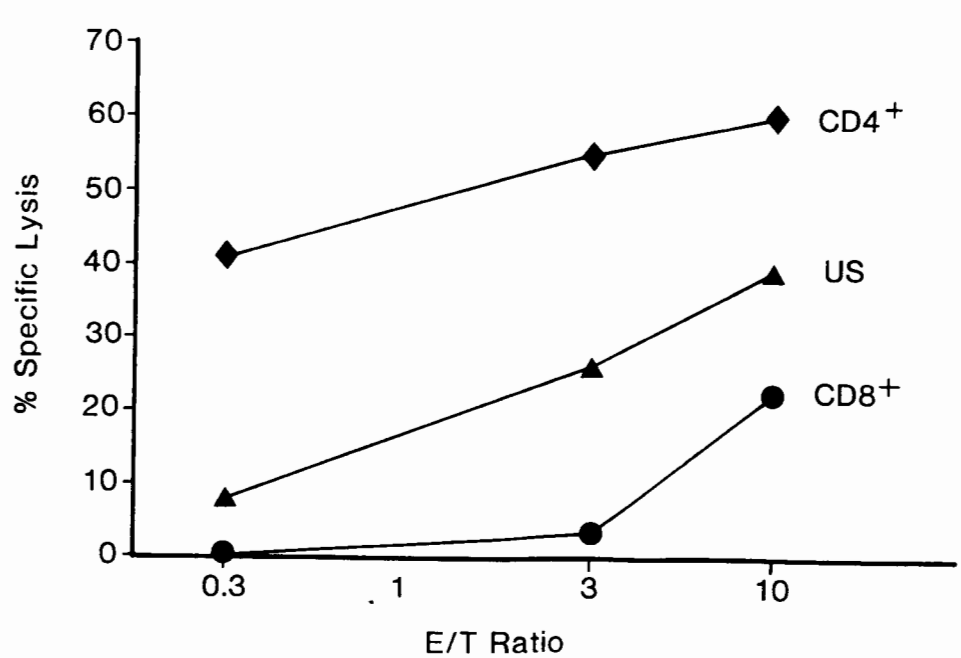


Table 4.4. Cytotoxicity by CD4 enriched lymphocyte populations.

		PERCENT SPECIFIC LYSIS OF MONOCYTES PULSED WITH:	
Experiment	E/T ratio	PPD	NO ANTIGEN
1	10	60	4
	3	49	14
	0.3	36	8
2	10	60	4
	3	55	0
	0.3	41	0

Lymphocytes were enriched for the CD4 subset by a panning technique and assayed for cytotoxic capacity in a 15-hour ^{51}chromium release assay.

Results are expressed as the mean percentage specific lysis of triplicate cultures calculated as described in Materials and Methods. SD were mostly less than 10%.

Table 4.5. Specificity and kinetics of PPD-stimulated cytotoxic activity.

		NATURE OF TARGET CELLS/ANTIGEN						
			AUTOLOGOUS MONOCYTES			ALLOGENEIC MONOCYTES		
Day	Effector	K562	No Ag	PPD	SK-SD	No Ag	PPD	SK-SD
1	PBL	10	2	4	2	2	2	2
	PEL	16	2	6	4	2	3	<2
4	PBL	304	3	22	4	<2	8	<2
	PEL	73	5	61	6	2	29	<2
6	PBL	69	5	31	4	ND	ND	ND
	PEL	26	<2	31	<2	ND	ND	ND

Comparison of cytotoxicity by PPD-stimulated effectors are shown after 1, 4, and 6 days of culture. NK activity was measured in a 4-hour chromium release assay. Results are expressed as lytic units/10^6 effector cells as stated in Methods. The tissue typing of the donor of the effector cells was A26,w33,B14,w42,DRw18,w14,DQw1,w4 and that of the donor of the heterologous monocytes was A1,24,B7,CW7,DR1,2, DQw1.

Ag=antigen

antigen-specific cytotoxicity was already present on day 1 of culture. (ii) In the pleural fluid an accelerated pattern of PPD-specific cytotoxicity was observed, peaking on day 4 of culture, as was seen in the 6 patients shown in Table 4.2. (iii) Despite the presence of significant NK-like activity against K562 target cells on day 4 and 6 of culture, the cytotoxicity which was observed in the 15-hour assay was both antigen-specific and HLA class 11 restricted. Significant kill was observed only on PPD-pulsed autologous and heterologous target cells sharing HLA-DQw1 with the donor, but not the heterologous target cells which were pulsed with the irrelevant antigen SK-SD, or no antigen. Additional evidence that the antigen non-specific kill was mediated by CD4 and CD8 negative NK or LAK cells was obtained from cytotoxicity assays employing enriched effector cell populations. Antigen non-specific kill was almost totally abrogated in the CD4 enriched effector cell population (Table 4.4). This contrasted strongly with the high levels of antigen specific kill seen in these assays (Fig. 4.3 and Table 4.4).

4.5. DISCUSSION AND CONCLUSIONS

These results indicate that antigen-specific cytotoxicity in PEL is significantly enhanced when compared to autologous and control PBL. In addition, accelerated kinetics of induction were observed with high levels of cytotoxicity already present by day 4. These findings, taken together with the clinical observation that most tuberculous pleural effusions resolve spontaneously, strongly implicate an *in-vivo* role for CTL in protection against mycobacteria. They also provided support for the current hypothesis that CTL are important in protection against mycobacterial infections (12). This hypothesis is attractive on two counts. Firstly, it would provide a mechanism for the destruction of

parasitized macrophages and secondly, the lysis of macrophages would prevent excessive suppression since it is known that macrophages are capable of suppressing the immune response in TB (41).

It is known that pleural effusions are enriched for PPD responsive $CD4^+$ cells, particularly those of the memory phenotype (138-141). These probably represent a heterogeneous population of cells, containing both helper and cytotoxic subsets. In the presence of active infection, these cells are likely to be *in-vivo* activated. Indeed, spontaneous proliferation in lymphocytes obtained from several patients with tuberculous pleuritis has been documented (chapter 7). The high peak levels of cytotoxicity obtained upon *in-vitro* stimulation of PEL with PPD, together with the accelerated kinetics of induction seen, strongly resembles secondary CTL responses obtained in immunized mice (142). This indicates that PPD-specific CTL have already been generated *in-vivo*, and it is likely, therefore, that the $CD4^+$ memory PEL are also enriched for precursor CTL. Available markers cannot differentiate helper from CTL within the $CD4^+$ subset, and limiting dilution analysis would be required to measure precursor frequencies of PPD-specific CTL in PEL (143).

The data also clearly demonstrates a dissociation between proliferative and cytotoxic responses. No significant antigen-specific cytotoxicity was generated on day 4 by patient 1 in Table 3 in spite of a vigorous proliferative response (LTI=72). This is likely to be due to a relatively low frequency of antigen-specific precursor cytotoxic cells in this non-tuberculous effusion, since significant cytotoxicity was present by day 6 of culture. Similarly, preliminary observations in this laboratory indicate that only low levels of PPD-specific cytotoxicity are generated by synovial T lymphocytes, obtained from patients with

rheumatoid arthritis, in spite of proliferative responses comparable to that found in tuberculous pleural effusions. These observations are compatible with previous reports indicating that the requirements for proliferation, and generation of cytotoxicity, are different (144,145).

The PPD-specific kill observed on day 6 in two patients with non-tuberculous effusions probably reflected previous infection or recent exposure without active infection. This contrasted strongly with our observations in active infection where *in-vivo* priming resulted in accelerated secondary responses observed *in-vitro*.

It has previously been shown that PPD-stimulation of PBL (from normal donors as well as patients with leprosy or pulmonary tuberculosis) results in the generation of both antigen-specific cytotoxicity, as well as antigen-independent killing (46-48). The latter has been attributed to the effects of $CD16^+$ NK and LAK cells (46), and also occurs in mixed lymphocyte cultures. Although strong NK-like activity was demonstrated against K562 target cells in 4-hour chromium release assays, cytotoxicity which was observed in simultaneously performed 15-hour chromium release assays, was significantly antigen-specific. It was also major histocompatibility complex-restricted (Table 4.5), as has been reported in patients with leprosy (46). Furthermore, subset fractionation experiments showed that lysis of PPD-pulsed monocytes was mediated almost exclusively by $CD4^+$ cells, in agreement with previous reports (46,47). Indeed, using the identical culture system as employed here, it has recently been shown that stimulation by PPD results in the generation of $CD4^+16^-$ cytotoxic effector cells (48). We found that antigen non-specific kill was also almost totally abrogated in the $CD4^+$ subset (Table 4.4), consistent with the finding that antigen non-specific killers are negative for both CD4 and CD8

markers. It is therefore likely that antigen non-specific cytotoxicity was mediated by LAK cells.

The data shown here may also have diagnostic and therapeutic implications. Firstly, the observation of accelerated proliferative and cytotoxic cellular immune responses only in patients with tuberculosis suggests a potential diagnostic role. The sensitivity and specificity of these observations are further evaluated in chapter 6. Secondly, conventional anti-tuberculous therapy is prolonged and compliance is frequently a major problem, and patients with multiple resistance to anti-tuberculous drugs frequently require prolonged hospitalization. Some patients also have persistent antigen secretion by circulating adherent cells long after full anti-tuberculous therapy (chapter 2). It may be possible to accelerate recovery in these patients if *in-vivo* cytotoxicity can be boosted by the administration of subcutaneous lymphokines. This has already been achieved in patients with Leprosy (46).

CHAPTER 5

FAILURE TO DEMONSTRATE PPD OR BCG-STIMULATED CD8 MEDIATED CYTOTOXICITY BY THE INTRODUCTION OF PPD OR BCG INTO THE CLASS I PATHWAY OF ANTIGEN PRESENTATION

5.1. ABSTRACT

Despite theoretical considerations, experimental confirmation of PPD-specific CD8 mediated cytotoxicity has not been forthcoming in humans. One possible explanation for this difficulty is that *in-vitro* exogenously added antigen enters only the class II pathway of antigen processing. The activation of $CD8^+$ CTL requires antigen to be presented in the context of MHC class I. This has been experimentally realized by the introduction of soluble protein directly into the cytoplasm by using osmotic changes to lyse antigen containing pinosomes. This technique was employed to determine if PPD or BCG-specific $CD8^+$ CTL could be generated. A 4-hour chromium release assay utilizing adherent cells targets was used to measure cytotoxicity. By using these techniques, no significant lysis could be demonstrated.

5.2. INTRODUCTION

Having demonstrated CD4 mediated lysis (chapter 4), evidence for CD8 mediated lysis was sought since experimental evidence for CD8 mediated cytotoxicity is lacking in humans (12,53).

$CD8^+$ CTL recognize antigen in association with MHC class I products. Thus the failure to demonstrate CD8 mediated cytotoxicity *in-vitro* may be due to the fact that exogenously added antigen enters the MHC class II pathway of

antigen processing. Recently it has been shown that exogenously added soluble protein can be made to enter the class I pathway of antigen presentation by the introduction of antigen directly into the cytoplasm of cells (55). This can be achieved by relatively brief (10 minutes) exposure of APC to antigen containing hypertonic medium (55). This results in the uptake of antigen in hypertonic medium into pinosomes (55). The pinosomes can then be lysed by returning the APC to isotonic medium (55). By this technique, class I restricted antigen presentation has been demonstrated after 6 hours (55). Class I restricted antigen presentation can be observed even sooner if APC are exposed to hypotonic medium immediately after exposure to antigen containing hypertonic medium since this causes osmotic rupture of pinosomes and "cytoplasmic loading" (55). Targets cytoplasmically loaded with antigen have been shown to express class I associated antigen for up to 2 days (55).

In this section the attempted generation of $CD8^+$ CTL, by the introduction of PPD or BCG directly into the MHC class I pathway of antigen presentation, is discussed.

5.3. MATERIALS AND METHODS

5.3.1. Generation of PPD or BCG-primed CD8+ effectors

PPD (5 mg/ml or 30mg/ml, Connaught laboratories) or BCG (30mg/ml, State Vaccine Institute, R.S.A., 20-40 organisms/ml) was dissolved in hypertonic medium made up as follows: 0.5M sucrose (BDH 10274, BDH LTD, Poole, England) 10% w/v polyethylene glycol 1000 (BDH 29544) and 10mM Hepes buffer (1M; pH 7.2; Flow 16-884-49; Flow Laboratories LTD, Irvine, Ayrshire, Scotland) in RPMI 1640 medium. The relatively high concentrations of antigen were chosen because previous data indicated that cytotoxicity was

significantly lower when less than 3 mg/ml of antigen were used for cytoplasmic loading (55). PBMC obtained from known PPD responsive control subjects were plated in 12 well sterile tissue culture plates (Nunc, Kamstrup, Denmark) at a concentration of 1 x 10^6/well. After 1-2 hours the NAC were removed from each well individually and the adherent cells were washed gently 3 times with PBS. Prewarmed PPD or BCG containing hypertonic medium was added to each well of adherent cells and incubated for 10 minutes at 37°C. Some adherent cells were exposed to the same dose of PPD or BCG in isotonic complete medium. The adherent cells were then washed 4 times with PBS and the corresponding NAC were added back. The cultures were incubated for 6 days at 37°C in a humidified atmosphere containing 5% CO_2.

5.3.2. Preparation of targets by osmotic lysis of pinosomes

Target cells were day 6 autologous adherent cells of PBMC that had been plated in 96-well U-bottomed tissue culture plates (Nunc, Kamstrup, Denmark). Approximately 10% adhered. This number was used for calculating E/T ratios. Approximately 18 hours prior to the assay, 100µl of varying concentrations of prewarmed PPD containing hypertonic medium (as prepared above) was added to the target cells and incubated for 10 minutes. Some target cells were not exposed to hypertonic medium. These targets were labelled with the same dose of PPD or BCG in the conventional way (i.e. in isotonic complete medium). The targets were then washed 4 times with prewarmed PBS and labelled with 6 uCi of ^{51}sodium chromate (specific activity 3.7-13Gq/mg, Amersham International, Amersham, UK) in isotonic complete medium and incubated at 37°C in a humidified atmosphere containing 5% CO_2 until used in the assay approximately 18 hours later.

5.3.3. Cytotoxic assay

The targets were washed 3 times with prewarmed complete medium and effectors were added in triplicate to the target cells at varying E/T ratios (10:1, 3:1 and 1:1). Wells with medium only were used for the determination of spontaneous release. Some plates were spun at 150G for 5 minutes. The assays were then incubated at 37^0C in a humidified atmosphere containing 5% CO_2. 4 hours later the total supernatant content of each well was transferred to a disposable counting tube (Greiner, Nurtingen, West Germany) and 100µl of triton-X was added to the remaining adherent cells for the determination of maximum release. After 3 hours the total volume of triton-X was transferred to similar tubes and the samples were counted in a gamma counter (Packard, auto-gamma scintillation spectrometer). The percentage-specific killing for each individual well was calculated as follows: percent specific lysis = [test cpm/(test cpm + cpm after triton-X treatment of the same well)] x 100% - percent spontaneous release. The percentage spontaneous release was calculated as follows: cpm in spontaneous release well/(cpm in spontaneous release well + cpm after triton-X treatment of the same well) x 100%.

5.4. RESULTS

5.4.1. Activation of effector cells

By using the technique described above, effector cells were successfully activated as determined by inverted phase microscopy. Numerous activated blasts were also observed under light microscopy.

5.4.2. Failure to demonstrate significant cytotoxicity

Only low levels of cytotoxicity were generated in this system (Table 5.1, 5.2, 5.3, 5.4). By contrast, the

Table 5.1. Cytotoxicity, against autologous adherent targets pulsed with PPD by cytoplasmic loading, in a 4-hour chromium release assay.

	PERCENT SPECIFIC LYSIS OF:	
	Targets cytoplasmically loaded with PPD by osmotic lysis of pinosomes	Targets subjected to osmotic lysis of pinosomes without PPD
EFFECTORS PRIMED BY APC CYTOPLASMICALLY LOADED WITH PPD:		
EXP.1: PPD 5mg/ml	14	3
EXP:2: PPD 30mg/ml	8	1
EFFECTORS CONVENTIONALLY PRIMED WITH PPD:		
EXP.1: PPD 5mg/ml	16	3
EXP.2: PPD 30mg/ml	0	0

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%.
All experiments were performed at an effector to target ratio of 10:1

Table 5.2. Cytotoxicity, against autologous adherent targets conventionally pulsed with PPD, in a 4-hour chromium release assay.

	PERCENT SPECIFIC LYSIS OF TARGETS PULSED WITH:	
	PPD	No antigen
EFFECTORS PRIMED BY APC CYTOPLASMICALLY LOADED WITH PPD:		
EXP.1: PPD 5mg/ml	12	11
EXP:2: PPD 30mg/ml	9	0
EFFECTORS CONVENTIONALLY PRIMED WITH PPD:		
EXP.1: PPD 5mg/ml	11	7
EXP.2: PPD 30mg/ml	7	0

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%.
All experiments were performed at an effector to target ratio of 10:1

Table 5.3. Cytotoxicity, against autologous adherent targets pulsed with BCG by cytoplasmic loading, in a 4-hour chromium release assay.

	PERCENT SPECIFIC LYSIS OF:	
	Targets cytoplasmically loaded with BCG by osmotic lysis of pinosomes	Targets subjected to osmotic lysis of pinosomes without BCG
EFFECTORS PRIMED BY APC CYTOPLASMICALLY LOADED WITH BCG:	4	6
EFFECTORS CONVENTIONALLY PRIMED WITH BCG:	7	4

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%.
All experiments were performed at an effector to target ratio of 10:1

Table 5.4. Cytotoxicity, against autologous adherent targets conventionally pulsed with BCG, in a 4-hour chromium release assay.

	PERCENT SPECIFIC LYSIS OF TARGETS PULSED WITH:	
	BCG	No antigen
EFFECTORS PRIMED BY APC CYTOPLASMICALLY LOADED WITH BCG:	15	0
EFFECTORS CONVENTIONALLY PRIMED WITH BCG:	18	7

Results are expressed as the mean percentage specific lysis of triplicate cultures as described in materials and methods. SD were mostly less than 10%.
All experiments were performed at an effector to target ratio of 10:1

percentage specific lysis at the same E/T ratio (10:1), using conventionally stimulated effectors and targets conventionally pulsed with 10 ug/ml PPD, increased to 35% in a parallel 15-hour chromium release assay (exp. 2 in Table 3.4).

No significant differences in percentage lysis was observed when conventionally primed effectors, or effectors primed with APC cytoplasmically loaded with PPD, were directed against targets cytoplasmically loaded with PPD (Table 5.1). Similarly, no significant differences were observed when either effector was directed against conventionally pulsed targets (Table 5.2).

5.5. DISCUSSION AND CONCLUSIONS

The data indicates that only low levels of PPD-specific cytotoxicity were observed in 4-hour chromium release assays. This was the case even when APC, both used as targets and for the priming of effectors, were subjected to osmotic lysis of pinosomes. This procedure, theoretically at any rate, ought to have resulted in the introduction of antigen directly into the cytoplasm, and therefore into the class I pathway of antigen presentation. These observations, therefore, suggest that $CD8^+$ CTL were not significantly generated in this system.

This conclusion is based on the assumption that $CD8^+$ cells would be responsible for any significant cytotoxicity over and above that observed by conventionally primed effectors and targets in 4-hour chromium release assays. The rationale for this assumption was a series of prior experiments demonstrating that $CD4^+$ CTL only showed low levels of lysis at 4 or 7 hours (Table 3.4). Peak cytotoxicity was, in fact, only observed at 15 hours (Table 3.4). On the other hand,

$CD8^+$cells were shown to be responsible for the cytotoxicity measured in a 4-hour chromium release assay, when targets were pulsed by using the technique of cytoplasmic loading by osmotic rupture of pinocytic vesicles (55).

The failure to demonstrate PPD-specific $CD8^+$ CTL may indicate that they are not important in the immune response to mycobacterial infection. No firm conclusions can be drawn, however, since no positive control which unequivocally demonstrated class I association of antigen was available in these experiments. The successful activation of effector T lymphocytes by exposure to APC cytoplasmically loaded with PPD or BCG (5.4.1) does not unequivocally demonstrate that antigen entered into the class I pathway of antigen presentation. It is likely that antigen also entered the class II pathway in spite of osmotic lysis of pinocytic vesicles since peripheral blood monocytes are rich in class II expression (52). The inability to demonstrate $CD8^+$ CTL may still, therefore, be due to the failure to introduce sufficient antigen into the class I pathway of antigen presentation.

Other potential explanations for the failure to generate $CD8^+$ CTL exist. In view of the fact that ovalbumin had already been successfully introduced into the class I pathway in previous animal studies (55), an attempt was made to use ovalbumin primed CTL directed against autologous ovalbumin labelled targets as a positive control. Unfortunately, this was not successful because control subjects showed very poor responses to ovalbumin.

In previous studies, tryptic digests of native ovalbumin were unable to sensitize $CD8^+$ CTL for lysis while Cnbr digests of the same antigen were successfully used to sensitize $CD8^+$ CTL (55). The nature of PPD and BCG may preclude entry into the class I pathway of antigen

presentation and may, therefore, be responsible for the failure to generate $CD8^+$ CTL.

In tuberculosis, $CD8^+$ CTL have been generated only in animal studies using bone marrow macrophages as targets and it is thought that this may be due to the unique target cells employed (54). The studies describing the introduction of antigen into the class I pathway of antigen presentation by osmotic lysis, used the class II negative target EL4 (55). It is therefore, also possible that CD8 mediated cytotoxicity is dependent on the nature of the target cells employed.

CHAPTER 6

THE KINETICS OF PPD STIMULATED LYMPHOCYTE PROLIFERATION: A COMPARISON OF PLEURAL EFFUSION LYMPHOCYTES AND AUTOLOGOUS PERIPHERAL BLOOD

6.1. ABSTRACT

The diagnosis of tuberculous pleuritis is based on pleural biopsy and culture. These techniques are invasive and not always successful. In this section, results of *in-vitro* proliferative responses of PEL and corresponding PBL to PPD and SK-SD were assessed on day 3 and day 6 of culture by [^{3}H]thymidine incorporation. PEL from a large number (65%) of patients with tuberculous pleuritis had an accelerated proliferative response to PPD. The finding was also specific in that no patients with non-tuberculous effusions demonstrated the phenomenon. These findings may be of diagnostic significance in patients with tuberculous pleuritis.

6.2. INTRODUCTION

Pleural biopsy is an invasive procedure not without complications (146). Suitable material for histology is not always obtained and culture of mycobacteria for diagnosis takes six weeks. Furthermore, it has been demonstrated that in 20-40% of patients with tuberculosis, and 40-60% with neoplasia, a definitive diagnosis cannot be made after closed pleural biopsy (146-149). Thus cellular immune studies which yield diagnostic information would almost certainly be useful in the clinical setting.

Having noted accelerated kinetics of proliferation and cytotoxicity in a small group of patients with tuberculous

pleuritis (chapter 4), this study was undertaken to determine the extent of the phenomenon. Because the phenomenon was noted to be unique to tuberculous pleuritis, the specificity and sensitivity was evaluated with respect to its diagnostic significance.

Several studies have examined cellular immune responses in pleural effusions (138-141,150,151). These studies have documented a significantly greater proliferative response in the pleural effusion than in the corresponding peripheral blood (138,139,150,151). None have, however, studied the kinetics of the proliferative response to PPD. Spontaneous lymphocyte proliferation has also not been systematically studied in pleural effusions. Following on our observations in the peripheral blood (chapter 2), the phenomenon was studied in tuberculous pleuritis.

6.3. MATERIALS AND METHODS

6.3.1. Patient population

Pleural fluid and peripheral blood samples were simultaneously obtained at the time of closed pleural biopsy in 31 patients with newly diagnosed tuberculous pleural effusions, seen at Groote Schuur Hospital between May and December 1989. Only cases with proven diagnosis (culture positive or positive pleural biopsy) were included in this group. Pleural fluid was also obtained from 13 patients with non-tuberculous aetiologies (malignancy, 10 cases; non-specific inflammation, 1 case; parapneumonic effusion, 1 case; pulmonary infiltrate and eosinophilia, 1 case).

6.3.2. Proliferation assay

PBL or PEL were separated on a Ficoll-Hypaque gradient and washed three times in PBS. PBL or PEL (1×10^5) were added to wells of 96 well round bottomed sterile tissue culture plates (Flow Laboratories) in 0.2 ml of complete medium. Cells were stimulated with 3µg/ml PPD (Connaught Laboratories, Willowdale, Ontario, Canada) or SK-SD (1:200 dilution, Lederle Lab., Wayne, N.J., U.S.A.). Some wells were left unstimulated for the determination of background proliferation. Cultures were incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 and pulsed with Tritiated thymidine (2 µCi/well, Amersham, U.K.) either on day 3 or day 6 for the final 16-18 hours of incubation. The cells were harvested using an automated harvester (Titertek 630), and radioactivity was measured using a liquid scintillation counter (Packard, Tricarb 4640). All assays were performed in triplicate. Results were expressed as difference in counts per minute (cpm) between antigen stimulated and unstimulated cells. A count of greater than 3000 cpm was considered a significant response (approximately the sum of the mean and 3 standard deviations of 22 unstimulated normal controls).

6.3.3. Statistical analysis

Analysis of variance by comparison of means was used to calculate P values.

6.4. RESULTS

6.4.1. Spontaneous lymphocyte proliferation in tuberculous effusions

Twelve of 31 patients had significant ($P < 0.01$) spontaneous lymphocyte proliferation in the PEL (Table 6.1). Five of these patients had significant ($P < 0.01$) spontaneous

Table 6.1. Spontaneous lymphocyte proliferation in patients with tuberculous pleuritis.

	PBL		PEL	
PATIENT	DAY 3	DAY 6	DAY 3	DAY 6
K.P.	1814	931	813	12731
C.C.	1571	12430	30926	37015
F.G.	3731	37368	7611	28348
A.S.	2595	31809	10242	8823
K.D.	501	4196	4470	12974
M.H.	1038	647	3103	3586
H.T.	743	2122	4698	4777
N.M	1569	1442	37222	5888
B.D.	1523	1519	3033	8081
B.K.	1073	1677	1953	3912
M.N.	614	1504	23063	28986
S.M.	2484	8223	5853	22847

Results are expressed as mean cpm of [^{3}H]thymidine incorporation by triplicate cultures of unstimulated lymphocytes in complete medium only. SD were mostly less than 10%. Results are shown for 12 out of 31 patients who were considered to have significant (greater than 3000 cpm) spontaneous lymphocyte proliferation in the pleural effusion.

lymphocyte proliferation both in the PEL and PBL (Table 6.1). Two of these patients had higher spontaneous lymphocyte proliferation on day 3, in the PEL, than on day 6 (patients A.S. and N.M. in Table 6.1). Of the remaining 19 patients 4 had significant ($P < 0.01$) spontaneous lymphocyte proliferation in the peripheral blood only (Table 6.2).

6.4.2. Spontaneous lymphocyte proliferation in non-tuberculous effusions

Spontaneous lymphocyte proliferation was not unique to tuberculous effusions. The phenomenon was seen in 8 of 13 non-tuberculous effusions (Table 6.3). The level of the proliferation was, however, much lower than that observed in tuberculous effusions (Table 6.1). Three patients had significant ($P < 0.01$) spontaneous proliferation both in the PBL and PEL (patients E.P., G.F. and R.M. in Table 6.3). Three patients had significant ($P < 0.01$) spontaneous proliferation in the PBL only (S.S., A.A. and C.A. in Table 6.3).

6.4.3. Response to PPD by PEL and PBL in patients with tuberculous pleuritis

Ten percent (3 of 29 patients) did not respond significantly in the PBL (patient A.W. in Table 6.4 and patients R.D. and S. M. in Table 6.5). These patients were all significantly positive in the PEL (Tables 6.4 and 6.5). Thirteen percent of patients (4 of 31) had negative responses on day 6 but were clearly positive on day 3 (Table 6.5).

6.4.4. Accelerated proliferative responses to PPD in tuberculous effusions

Twenty of 31 patients demonstrated accelerated proliferative responses to PPD in PEL with higher responses on day 3 as

Table 6.2. Patients with tuberculous pleuritis not showing significant spontaneous proliferation in the PEL.

	PBL		PEL	
PATIENT	DAY 3	DAY 6	DAY 3	DAY 6
B.L.	2040	1005	1285	444
T.M.	984	1209	2145	758
M.D.	1464	3607	1168	1066
A.F.	602	710	899	875
B.I.	1143	795	718	600
M.M.	800	1030	2006	878
A.G.	745	3962	1423	686
T.B.	994	7504	1037	1078
S.F.	684	3016	945	944
A.W	1800	1702	725	847
F.S.	599	2635	1048	982
S.N.	712	2340	1415	2070
D.K.	686	846	715	884
O.X.	713	1384	905	1754
J.D.	ND*	ND	1891	601
N.M.	675	1048	626	627
R.D.	982	550	1285	1114
F.F	ND	ND	940	870
J.M.	1298	1472	893	751

Results are expressed as mean cpm of [^{3}H]thymidine incorporation by triplicate cultures of unstimulated lymphocytes in complete medium only. SD were mostly less than 10%.

PBL data are presented for comparison.

*ND, not done.

Table 6.3. Spontaneous lymphocyte proliferation in patients with non-tuberculous effusions.

	PBL		PEL	
PATIENT	DAY 3	DAY6	DAY 3	DAY 6
T.M.	634	877	1918	1397
S.S.	1242	3083	533	487
H.P.	785	2495	872	968
A.A.	3591	10146	921	2781
C.A.	1261	10965	816	2870
N.S.	977	2192	8534	4357
E.P.	12345	1705	1935	13145
D.L.	1115	850	2410	6858
S.A.	1554	2122	21309	9563
G.P.	1202	761	7365	9494
N.S.	501	1307	4143	10616
G.F.	1290	3981	1982	5611
R.M.	700	14795	3455	4921

Results are expressed as mean cpm of [^{3}H]thymidine incorporation by triplicate cultures of unstimulated lymphocytes in complete medium only. SD were mostly less than 10%.

Table 6.4. Patients with tuberculous pleuritis not showing accelerated proliferative responses to PPD.

	PBL		PEL	
PATIENT	DAY 3	DAY 6	DAY 3	DAY 6
B.K.	12486	34422	98418	101011
B.L.	50082	107396	22532	24593
T.M.	2508	14093	57454	79657
M.D.	1644	28475	33438	68458
A.F.	703	11012	4144	7193
B.I.	1638	30901	44432	70819
M.M.	33586	217783	55793	94578
A.G	5675	57059	41576	44254
T.B.	2542	7643	15724	23554
F.S.	245	14656	49096	58757
A.W.	-372	1924	30012	43690

Results are expressed as difference in mean cpm, of [^{3}H]thymidine incorporation by triplicate cultures, between antigen stimulated and unstimulated wells. SD were mostly less than 10%. Results are shown for 11 out of 31 patients.

Table 6.5. Accelerated proliferative responses to PPD by PEL obtained from patients with tuberculous pleuritis.

	PBL		PEL	
PATIENT	DAY 3	DAY 6	DAY 3	DAY 6
S.F	2252	25568	20449	14951
S.N.	71724	61965	48464	25660
D.K.	8560	55298	68649	63214
O.X.	3363	28686	16258	521
J.D.	ND*	ND	48520	15875
N.M.	55917	33999	40964	21792
R.D.	827	1165	111023	34525
F.F.	ND	ND	58365	9778
J.M.	5553	21419	73335	37507
K.P.	5651	6660	14233	8696
C.C	7939	30272	90259	-19127
F.G.	7470	2174	69880	-23635
A.S.	52904	70712	139803	21886
K.D.	853	10674	39504	14067
M.H.	6208	6620	73432	13052
H.T.	5392	23219	49330	14629
N.M.	19028	43595	20679	13275
B.D.	27469	62013	88802	13664
M.N.	18345	161107	101973	21882
S.M.	1242	-1905	5419	-20351

Results are expressed as difference in mean cpm, of [^{3}H]thymidine incorporation by triplicate cultures, between PPD stimulated and unstimulated wells. SD were mostly less than 10%. Results are shown for 20 of 31 patients who demonstrated higher proliferative responses, in the PEL, on day 3 than on day 6.

*ND, not done.

compared to day 6 (Table 6.5). Two of these patients also had accelerated responses in the PBL (S.N. and F.G. in Table 6.5). By contrast, none of the patients with non-tuberculous effusions had an accelerated response to PPD in the PEL (Table 6.6). Only one patient had an accelerated response in the PBL. The accelerated responses were antigen-specific since the response to SK-SD demonstrated conventional kinetics in all patients tested (Table 6.7).

6.5. DISCUSSION AND CONCLUSIONS

Tuberculosis is a disease almost entirely regulated by the cell-mediated immune response of the host (18). It is, therefore, not unreasonable to expect understanding of the cellular immune response to provide information of diagnostic significance.

Three unique observations about the cell-mediated immune response are evident from the results in this section. Firstly, a subset of patients demonstrate significant spontaneous lymphocyte proliferation in the pleural effusion (Table 6.1). Secondly, a large number (65%) of patients with tuberculous pleuritis demonstrate an accelerated proliferative response to PPD (Table 6.4). Thirdly, a negative or low proliferative response on day 6 may not indicate a negative result, but that the *in-vitro* response had already peaked.

One effect of the spontaneous lymphocyte proliferation, was to reduce the sensitivity of lymphocyte transformation indeces. The indeces were falsely depressed by the high spontaneous proliferation. Difference in mean cpm between antigen stimulated and unstimulated wells was therefore utilized, with difference in cpm exceeding 3000 cpm being considered significant.

Table 6.6. Proliferative responses to PPD in patients with non-tuberculous effusions.

	PBL		PEL	
PATIENT	DAY 3	DAY 6	DAY 3	DAY 6
T.M.	632	2482	-549	-764
S.S	1228	8468	235	977
H.P.	433	101816	61661	73202
A.A	17320	10258	1903	2692
C.A	1042	-2092	-800	869
N.S.	15928	26599	9336	12548
E.P.	-2378	651	-392	31020
D.L.	2412	44669	5602	10873
S.A.	35022	46908	-10670	-6828
G.P.	-416	144	-1664	4978
N.S.	749	2324	2591	-7947
G.F.	11547	102901	19441	213301
R.M.	441	2095	4087	5913

Results are expressed as difference in mean cpm, of [^{3}H]thymidine incorporation by triplicate cultures, between antigen stimulated and unstimulated wells. SD were mostly less than 10%.

Table 6.7. Proliferative responses to SK-SD in patients with tuberculous pleuritis.

	PBL		PEL	
PATIENT	DAY 3	DAY 6	DAY 3	DAY 6
S.F	879	1571	11	6986
S.N.	42739	49377	10890	14116
D.K.	1674	9764	1760	4184
O.X.	94	1807	304	219
R.D.	-332	2622	-56	-270
J.M.	-451	-324	-209	28
K.P.	-335	733	2313	23164
K.D.	945	6894	10123	14148
N.M.	-32518	35112	-32226	9513
B.D.	1584	50510	761	18601
S.M.	-1400	-6208	-681	-21304
B.K.	1093	20828	3459	54500
B.I.	988	3394	1017	7463
M.M.	12194	62423	12987	37217
A.G.	3241	42557	5513	55811
T.B.	5	-3526	648	6356
F.S.	2366	57840	-543	20515
A.W.	-109	1959	76	4369

Results are expressed as difference in mean cpm, of [^{3}H]thymidine incorporation by triplicate cultures, between antigen stimulated and unstimulated wells. SD were mostly less than 10%.

The spontaneous lymphocyte proliferation may either reflect *in-vivo* activation at the site of the pathology or persistent mycobacterial antigen secretion by antigen presenting cells (chapter 2). Because repeated samples of PEL cannot be obtained from patients with tuberculous pleuritis, further characterization of the phenomenon at this site was not possible.

An interesting observation was that all the patients with spontaneous lymphocyte proliferation had accelerated kinetics of proliferation to PPD. This suggests that the spontaneous proliferation is a manifestation of *in-vivo* activation. It follows, therefore, that the accelerated kinetics of proliferation is a secondary immune response.

The observation that PPD responses were negative on day 6 in some cases in the pleural effusion, but strongly positive on day 3, indicates the need to do kinetic studies when assessing the immune response in patients with tuberculous pleuritis. Furthermore, the observation that some patients had compartmentalized immune responses to PPD, (i.e. negative responses in the blood, but positive in the effusion) indicates the necessity of studying the immune response at the site of the pathology. Observations in the peripheral blood alone may be misleading.

The restriction of the accelerated proliferative responses to patients with tuberculous pleuritis underlines the diagnostic value of this observation. On the basis of this observation, an early diagnosis could have been made in 65% of patients with tuberculous pleuritis, using a relatively non-invasive procedure. It may even be possible to make a diagnosis within 3 days and 24 and 48 hour responses to PPD will have to be examined in this regard. Furthermore, it is likely that accelerated proliferative responses will also be observed at other sites such as ascites and pericardial

fluid. This may further extend the diagnostic usefulness of the observation.

CHAPTER 7

COMPARTMENTALIZATION OF THE CELLULAR IMMUNE RESPONSE TO THE RECOMBINANT 65 kDa HEAT SHOCK PROTEIN OF MYCOBACTERIUM BOVIS BCG IN PATIENTS WITH TUBERCULOUS PLEURITIS

7.1. ABSTRACT

Proliferative and cytotoxic responses to the recombinant 65 kDa antigen of *M. bovis* BCG was analyzed. PEL and autologous PBL obtained from patients with tuberculous and non-tuberculous pleuritis were stimulated *in-vitro* with the recombinant 65 kDa antigen. Proliferation was assessed by ^{3}[H]thymidine incorporation. In addition, PEL were activated *in-vitro* with the 65 kDa antigen and tested for CTL activity in a 15-hour chromium release assay. A high percentage (56%) of PEL obtained from patients with tuberculous pleuritis, but not non-tuberculous pleuritis, showed significant proliferative responses to the 65 kDa antigen. The response in autologous PBL was also significantly lower both in terms of number of patients responding and also intensity of the response. PEL activated with the 65 kDa antigen showed antigen-specific lysis of autologous targets. These results indicate compartmentalization of the immune response to the 65 kDa antigen of *M. Bovis* BCG, and in addition, provide evidence for *in-vivo* involvement of this antigen in the immune response to *M. tuberculosis*.

7.2 INTRODUCTION

The recombinant 65 kDa heat shock protein of *M. bovis* BCG has been shown to be immunodominant in mice immunized with *M. tuberculosis* (70). Antigen-specific and non-specific CTL have been generated against 65 kDa treated target cells (46). However little is known about reactivity, by patients with tuberculous pleuritis, to this antigen.

More recently, spontaneous proliferation by hybridomas obtained from neonatal thymocytes has been shown to be due to recognition of the 65 kDa HSP (90). Thus the spontaneous lymphocyte proliferation observed in peripheral blood and pleural effusions (chapters 2 and 6) may also be due to recognition of either mycobacterial HSP or the human homologue. Thus, reactivity to the the recombinant 65 kDa antigen of *M. bovis* BCG was studied in patients with tuberculous pleuritis.

7.3 MATERIALS AND METHODS

7.3.1. Patient population

Pleural fluid and peripheral blood samples were simultaneously obtained at the time of closed pleural biopsy in 18 patients with newly diagnosed tuberculous pleural effusions, seen at Groote Schuur Hospital between July and December 1989. Pleural biopsy revealed granulomatous pleuritis with the presence of *M. tuberculosis* in all cases. Pleural fluid was also obtained from 7 patients with non-tuberculous aetiologies (malignancy, 6 cases and non-specific inflammation, 1 case). Peripheral blood samples were also obtained from control subjects for the analysis of cytotoxicity. These controls were the same as those employed in chapter 6.

7.3.2. Proliferation assay

PBL or PEL were separated on a Ficoll-Hypaque gradient and washed three times in PBS. Cells (1×10^5) were added to wells of 96 well round bottomed sterile tissue culture plates (Flow Laboratories) in 0.2 ml of complete medium. Cells were stimulated with 5 µg/ml of the 65 kDa HSP of *M.*

Bovis BCG (a kind gift from Dr J. D. A. Van Embden, WHO IMLEPP programme) or 3µg/ml PPD (Connaught Laboratories, Willowdale, Ontario, Canada) except for some wells which contained responder cells alone for the determination of background proliferation. Cultures were incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 and pulsed with Tritiated thymidine (2 µCi/well, Amersham, U.K.) on day 3 and day 6 for the final 16-18 hours of incubation. The cells were harvested using an automated harvester (Titertek 630), and radioactivity was measured using a liquid scintillation counter (Packard, Tricarb 4640). All assays were performed in triplicate. Results were expressed as lymphocyte transformation indices (LTI). The LTI was calculated as follows: LTI = mean counts per minute in antigen stimulated wells/mean counts per minute in unstimulated wells. An LTI of greater than 3 was considered a significant response.

7.3.3. Cytotoxicity assay

The cytotoxic capacity of effector cells which had been stimulated for 6 days with the 65 kDa HSP of BCG in 24 well tissue culture plates (Nunc, Kamstrup, Denmark) was assayed as described in section 3.3.3. Target cells were labelled with 10 µg/ml 65 kDa antigen, 10µg/ml PPD or a 1:100 dilution of SK-SD. Some target cells did not receive antigen.

7.3.4. Statistical analysis

Statistical analysis was performed using Kruskal-Wallis one-way analysis of variance for continuous measurements. Categorical data was analyzed using chi-square or Fisher's exact test.

7.4 RESULTS

7.4.1. PPD and 65 kDa antigen reactivity in TB pleuritis.

Fig. 7.1 compares the responsiveness of PEL and PBL obtained from 18 patients with proven Tuberculosis, to stimulation by the recombinant 65 kDa antigen or PPD. Results are expressed as the percentage of patients whose lymphocytes demonstrate a significant response to antigen, defined as an LTI > 3. Fifty six percent (10 of 18) of tuberculous effusions tested demonstrated significant responses to the 65 kDa antigen (Fig. 7.1). Only 11% (2 of 18) of autologous PBL responded. This difference was statistically highly significant ($p < 0.003$). The response to PPD was much greater in both PEL (100%) and PBL (94%) (Fig. 7.1).

7.4.2. The intensity of the response to 65 kDa is greater at the site of the pathology.

The magnitude of the proliferative response by PEL and PBL to the 65 kDa antigen and PPD were compared. Reactivity was significantly higher in pleural fluid than in peripheral blood, with mean LTI $\pm$ standard error of 10.16 ± 3.16 vs. 1.6 ± 0.2, respectively ($n=18$, $p < 0.01$). While reactivity to PPD tended to be higher in pleural fluid than in the peripheral blood (mean LTI $\pm$ standard error of 50.3 ± 8.8 vs. 38.4 ± 12.8), this difference did not achieve statistical significance.

7.4.3. The 65 kDa antigen shows greater specificity when compared to PPD.

Lymphocytes from 3 of 7 non-tuberculous effusions showed reactivity to PPD (Table 7.1). By contrast none of the non-tuberculous effusions demonstrated significant reactivity to the 65 kDa antigen (Table 7.2). Similar results were obtained for PBL obtained from these patients (Table 7.2).

Fig. 7.1. Lymphocytes obtained from patients with Tuberculous pleuritis were stimulated with PPD or the recombinant 65 kDa mycobacterial antigen as shown on the x-axis. The percentage of patients showing a significant response (LTI > 3) is shown on the y-axis. The response by PBL or PEL effectors is indicated on the z-axis.

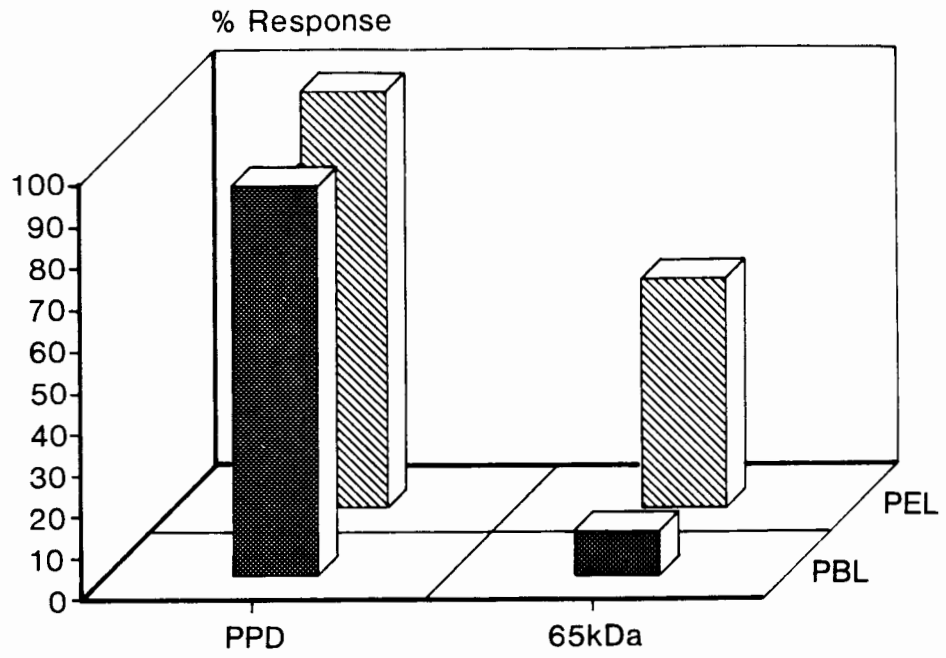


Table 7.1. Proliferative responses of PEL and PBL, obtained from patients with non-tuberculous effusions, to PPD.

	PBL		PEL	
Patient	DAY 3	DAY 6	DAY 3	DAY 6
1	10	27	11	39
2	0.6	1	0.7	1.5
3	6	2	3	2
4	19	42	72	77
5	17	13	4	6
6	1	1	1	3
7	3	54	3	2.5

The results are expressed as lymphocyte transformation indexes (LTI). The LTI was calculated as follows: mean counts per minute of [^{3}H]thymidine incorporation in antigen stimulated wells/mean counts per minute of [^{3}H]thymidine incorporation in unstimulated wells. An LTI of greater than 3 was considered a significant response.

Table 7.2. Proliferative responses of PEL and PBL, obtained from patients with non-tuberculous effusions, to the recombinant 65 kDa heat shock protein of *M. Bovis* BCG.

	PBL		PEL	
Patient	DAY 3	DAY 6	DAY 3	DAY 6
1	1	1	1	2
2	2.5	19	0.6	0.6
3	0.6	1	1,4	1
4	2.6	1.2	1.2	0.5
5	2.8	1.8	3	0.6
6	0.4	1	2	0.8
7	2	2.7	0.8	2.6

The results are expressed as lymphocyte transformation indexes (LTI). The LTI was calculated as follows: mean counts per minute of [^{3}H]thymidine incorporation in antigen stimulated wells/mean counts per minute of [^{3}H]thymidine incorporation in unstimulated wells. An LTI of greater than 3 was considered a significant response.

7.4.4. Reactivity to 65 kDa demonstrates conventional kinetics.

Because accelerated kinetics of proliferation were observed by PEL stimulated with PPD (chapter 6), the kinetics of the response to the 65 kDa antigen was determined. Reactivity to the 65 kDa antigen, however, demonstrated conventional kinetics (Table 7.3).

7.4.5. 65 kDa primed effectors demonstrated antigen specific cytotoxicity at the site of the pathology.

65 kDa labelled targets were significantly lysed by autologous 65 kDa primed effectors in all three patients tested (Table 7.4). PPD labelled targets were also significantly, although less efficiently, lysed (Fig.7.2). Some antigen non-specific kill was observed at high E/T ratios (Table 7.4, Fig.7.2 and Fig. 7.3). A similar pattern was seen in control PBL (Fig. 7.4).

7.5 DISCUSSION AND CONCLUSIONS

The 65 kDa HSP of BCG is a major immunogenic component of *M. Bovis BCG*, *M. Tuberculosis*, and *M. Leprae* and is frequently recognized by murine and human B cells and helper T cells (47,53,67-69). Studies in mice immunized with *M. tuberculosis* have demonstrated that 20% of mycobacteria-reactive T lymphocytes recognize this molecule (70). Amino acid sequence determination has revealed that the 65 kDa antigen is identical in *M. bovis* and *M tuberculosis*, and highly homologous to the *Eschericia coli* HSP GroEL (70,71). The human homologue has also been found to be 50% identical with the mycobacterial protein (72).

Table 7.3. Proliferative responses of PEL and PBL, obtained from patients with tuberculous pleuritis, to the recombinant 65 kDa heat shock protein of *M. Bovis* BCG.

	PBL		PEL	
PATIENT	DAY 3	DAY 6	DAY 3	DAY 6
1	0.5	3	1	3.5
2	1	2	2	26
3	2	1	5	7
4	1	1	1	16
5	2	3.7	1	1
6	2	2	2	2
7	2	3.5	10	50
8	2	1	3	29
9	1	0.2	1	1
10	3	1	2	1
11	1	0.6	0.8	0.5
12	1.6	0.2	1.5	2
13	ND	3	ND	6
14	1	1	6	11
15	1	1	3	5
16	1.5	2.5	1.5	1
17	1.1	1	5	20
18	1	2	1	1

The results are expressed as lymphocyte transformation indeces (LTI). The LTI was calculated as follows: mean counts per minute of [^{3}H]thymidine incorporation in antigen stimulated wells/mean counts per minute of [^{3}H]thymidine incorporation in unstimulated wells. An LTI of greater than 3 was considered a significant response.

ND, not done

Table 7.4. Mean cytotoxicty by 65 kDa stimulated PEL obtained from patients with tuberculous pleuritis.

		% SPECIFIC LYSIS OF TARGETS PULSED WITH:		
Assay type	E/T ratio	65 kDa	SK-SD[+]	NO Ag[*]
15-hour	10	19 ± 6	6 ± 2	5 ± 2
	3	10 ± 5	3 ± 1	3 ± 2
	0.3	8 ± 6	2 ± 2	2 ± 2

Data represent mean ± SE of cytotoxicity measured in 3 patients.

[+]SK-SD, streptokinase-streptodornase

[*]Ag, antigen

Fig. 7.2. Lysis of autologous adherent cell targets labelled with 65 kDa (♦), PPD (●), SK-SD (△) or unlabelled (▲) by 65 kDa stimulated PEL effector cells obtained from a patient with tuberculous pleuritis. Cytolysis was determined by chromium release.

PATIENT 1

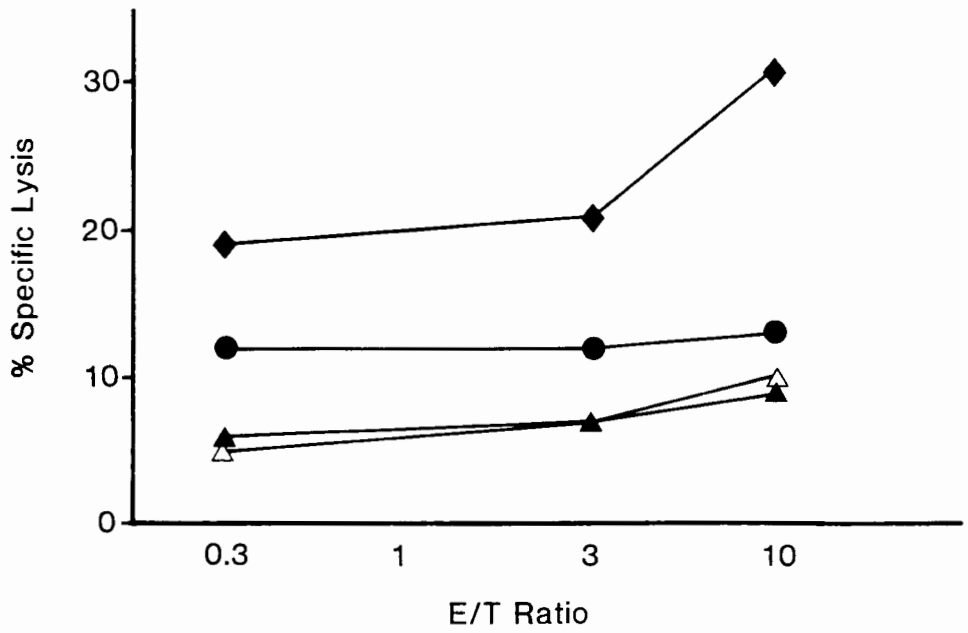


Fig. 7.3. Lysis of autologous adherent cell targets labelled with 65 kDa (♦), SK-SD (●) or unlabelled (△) by 65 kDa stimulated PEL effector cells obtained from a patient with tuberculous pleuritis. Cytolysis was determined by chromium release.

PATIENT 2

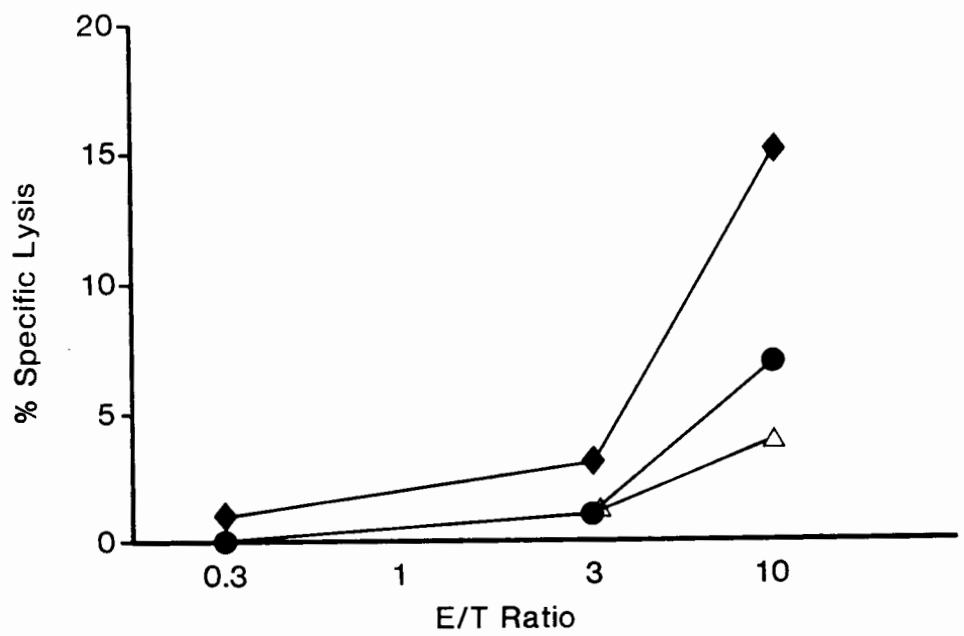
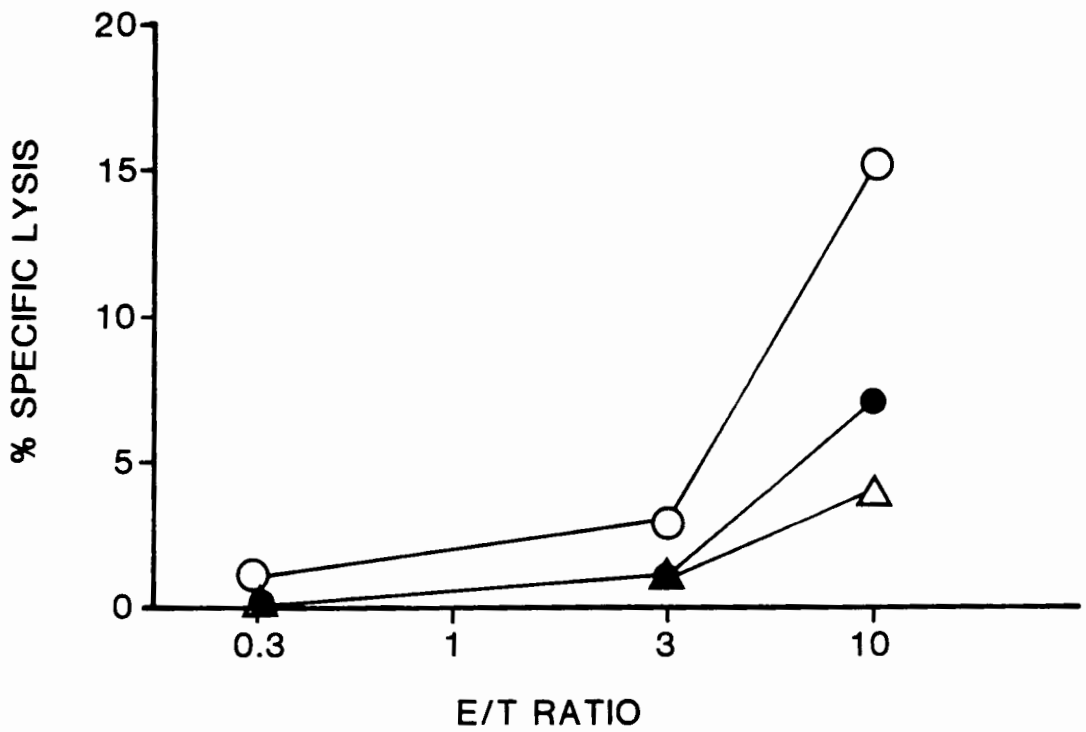


Fig. 7.4. Lysis of autologous adherent cell targets labelled with 65 kDa (○), SK-SD (●) or unlabelled (△) by 65 kDa stimulated PBL effector cells obtained from a normal control. Cytolysis was determined by chromium release.



It has, therefore, been considered a potential candidate for a subunit vaccine. Doubt about its widespread application as a vaccine arose when a study showed that the antigen was immunodominant in only 20% of 47 individuals tested (47). However, the above mentioned study was performed on cells obtained from peripheral blood (47). The findings contained herein demonstrate that, at the site of the pathology, a much higher percentage of patients (56%) are responsive to the 65 kDa antigen. In addition, it is shown that only 11% respond in the peripheral blood. The high response rate was specific to tuberculous pleural effusions since no reactivity was observed in seven non-tuberculous effusions, despite the fact that three of these patients responded to PPD (Table 7.2).

The much lower response to the 65 kDa antigen observed in the peripheral blood of patients with tuberculous pleuritis may reflect suppression since it is known that both monocytes and T cells may function as suppressor cells in tuberculous peripheral blood (41,42,151). The low response rate in the blood may, in fact, be beneficial to the host since the 65 kDa antigen has been implicated in autoimmune disease (72,75,76). Suppression in the PBL, therefore, may function to reduce the potential for widespread autoimmune reactions to occur.

Although limited to three patients, the finding that the 65 kDa antigen serves as a target for 65 kDa primed effector CTL (Table 7.4, Fig.7.2 and Fig. 7.3) is in agreement with a previous study (47). However, the 65 kDa primed PEL obtained from these patients did not demonstrate the high levels of antigen non-specific kill observed in other studies (47-48). Similarly, only low levels of antigen non-specific kill was observed in control peripheral blood (Fig. 7.3). One reason for this difference may be that the targets in the previous study were "stressed" in some way, since it has been

demonstrated that monocytes "stressed" by exposure to IFN-Ó express cross-reactive epitopes to the recombinant 65 kDa antigen (74). It has been shown that these cross-reactive epitopes can be presented to CTL with reactivity to the recombinant 65 kDa antigen (74).

In conclusion, these results demonstrate that PEL in a high percentage (56%) of patients with tuberculous pleuritis, respond to the 65 kDa antigen. In addition, PEL primed with the 65 kDa antigen demonstrated antigen-specific cytolysis. These findings confirm that 65 kDa antigen responsiveness is more widespread than has been previously thought, and suggests that it is a candidate for a subunit vaccine. Furthermore, the marked compartmentalization of the immune response seen here emphasizes the importance of evaluating lymphocyte responses at the site of the pathology.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

Several new findings are described in this thesis. These include: (1) persistent *in-vitro* spontaneous proliferation by peripheral blood and pleural effusion lymphocytes, (2) accelerated and augmented proliferative and cytotoxic cellular immune responses in tuberculous pleural effusions, and (3) enhanced reactivity to the recombinant 65 kDa antigen of *M. Bovis* BCG by pleural effusion lymphocytes. These observations substantiate the pivotal role played by cellular immune mechanisms in immunity following mycobacterial infection.

Several characteristics of the spontaneous proliferation implicated a role for APCs and antigen in its initiation. The exact nature of this antigen was not established but since the phenomenon was observed in patients with active tuberculosis, it is probable that the antigen was derived from mycobacteria. However, self-antigen cross-reactive with mycobacterial antigen could also account for the spontaneous proliferation. A known example of such an antigen is the 65 kDa HSP (74). Thus the finding that reactivity to the 65 kDa antigen correlated with active disease (chapter 7) could suggest that the antigen responsible for initiation of the spontaneous lymphocyte proliferation may indeed have been mycobacterial 65 kDa HSP or the human homologue. Consequently, future research will be aimed at isolating the spontaneously proliferating cells, and determining the nature of the T cell receptors employed and their antigen reactivity. It has been shown that the gamma-delta T cell receptor was exclusively used by spontaneously proliferating hybridomas obtained from neonatal thymocytes (90). These hybridomas were shown to respond to the 65 kDa HSP, but not other bacterial antigens, thus indicating that the

spontaneous proliferation was a response to the 65 kDa HSP (90).

Spontaneous lymphocyte proliferation would be significant irrespective whether due to endogenous human HSP or persisting mycobacterial antigen. The significance of the former is that it could account for destruction of self tissue even in the absence of ongoing infection, since it is evident that the 65 kDa antigen can prime CTL and also serve as a target for such CTL (Chapter 7). The significance of the latter is that persisting antigen (whether 65kDa or other mycobacterial antigen) would represent a failure by cell mediated immunity to clear infected macrophages. This would therefore imply a failure of the candidate mechanisms employed in the clearance of parasitized macrophages i.e. IFN-Ó mediated macrophage activation, cytokine mediated macrophage destruction and/or cell mediated macrophage lysis. For reasons discussed earlier in this work, cell mediated cytolysis was considered the most important mechanism. Thus it was postulated that defective cell mediated cytolysis could have accounted for the failure to clear infected macrophages in the patients with spontaneous lymphocyte proliferation. As a result of technical difficulties (including particularly ill patients who could not be repeatedly venesected for the provision of target cells and patients being lost to follow up) this hypothesis could not be pursued further in the patients with spontaneous proliferation. Nevertheless, the general hypothesis that cell mediated cytotoxicity was important for protection against mycobacteria was explored.

Clear evidence was provided of augmented and accelerated PPD-specific cytotoxicity in pleural effusion lymphocytes (Chapter 4). Since this resembled a secondary immune response, the implication was that cytotoxic T cells were being primed *in-vivo*. This is the first evidence implicating

an *in-vivo* role for cytotoxic T cells in human tuberculosis. Thus caseation within tuberculous granulomata may indeed result from the effects of CTL activity. Furthermore, antigen non-specific kill was also demonstrated and found to be likely to be due to NK activity (Chapter 4). The significance of this finding is that it may represent another mechanism responsible for tissue damage in pulmonary tuberculosis.

The PPD-specific cytotoxic cells were found to be $CD4^+$ and MHC class II restricted (Chapter 4). Attempts to generate $CD8^+$ CTL were not successful but no firm conclusions could be drawn since a positive control was lacking (chapter 5). However, the failure to unequivocally demonstrate PPD-specific CD8 mediated cytotoxicity was not unique as can be seen from the literature. It is possible that these observations reflect the actual *in-vivo* requirements for protection since *M. Tuberculosis* is not usually seen in cells other than professional phagocytes (13). Thus it may be that *M. Tuberculosis* exhibits a tropism for these cells and does not infect host parenchymal cells. Professional phagocytes carry the class II antigen and can therefore be recognized by $CD4^+$ CTL (52). An alternative explanation is that host parenchymal cells die soon after becoming parasitized with *M. Tuberculosis*, due to enhanced TNF sensitivity, and thus there may be no need for CD8 mediated cytotoxicity (13). The requirements in Leprosy on the other hand may be quite different since the bacterium is often seen in parenchymal cells (13). These cells usually only express the class I antigens and therefore are recognized by $CD8^+$ CTL.

Reactivity to the 65 kDa protein was found to be compartmentalized and to correlate with active disease (Chapter 7). Antigen-specific cytotoxicity by CTL primed with the recombinant 65 kDa protein of *M. Bovis* BCG was also

found to be directed against target cells treated with both the 65 kDa protein and PPD (Chapter 7). These findings serve to further confirm the importance of this antigen in immunity to tuberculosis and suggest that it may form a major component of a future vaccine.

The accelerated proliferative response by PPD-stimulated lymphocytes was further analyzed for diagnostic value (Chapter 6). Sixty five percent of unselected tuberculous effusions were shown to have accelerated kinetics of proliferation. All of these were subsequently confirmed to have tuberculosis. The phenomenon was not seen in non-tuberculous effusions and therefore has diagnostic value. These observations have already been extended to pericardial effusions and future efforts will be directed at ascites. The small yield of lymphocytes obtained from cerebrospinal fluid is likely to prove a major limitation in the setting of tuberculous meningitis. It would be important to determine if the assay could be microtized since the diagnosis of tuberculous meningitis remains a difficult clinical problem. Cellular assays are unfortunately expensive, labour intensive and require highly trained personnel. The clinical application for this test may therefore be limited to selected cases where a diagnosis cannot be made with more conventional tests.

The observation that PPD-specific cytotoxicity is augmented and accelerated in tuberculous pleural effusions raises questions about cell mediated cytotoxicity in other clinical forms of tuberculosis. Thus it would be interesting to document this in future studies. For example, preliminary observations suggest that cytotoxicity is reduced in disseminated tuberculosis. It would be important to determine if the cytotoxicity could be enhanced by the administration of exogenous cytokines as has been demonstrated in some patients with Leprosy (46). Studies

would also have to be conducted to determine if such manipulation of the cytotoxic response resulted in clinically beneficial responses.

Numerous complications exist with current chemotherapeutic regimes. Treatment lasts for 6-9 months and compliance is consequently a major problem. Drug related toxicity is a major limitation and multi-drug resistant strains of *M. tuberculosis* are also increasingly being encountered. Thus there is little doubt that additional modalities of therapy such as immunomodulation will be required to combat tuberculosis particularly in the AIDS era.

CHAPTER 9

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